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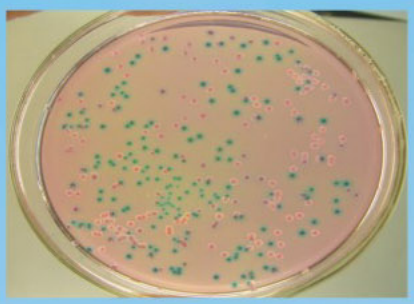


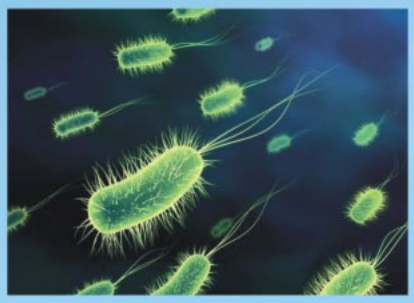
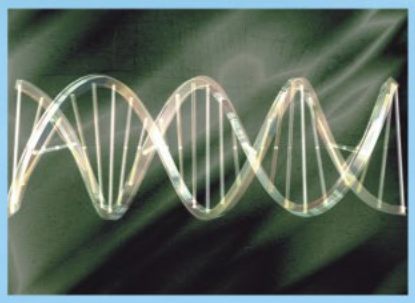

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Interdisciplinary Researches

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Phenotypic Characterization of Indigenous Iraqi *Sinorhizobium meliloti* Isolates for Abiotic Stress Performance

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Abstract: Alfalfa (*Medicago sativa* L.) is being grown in harsh environment in Iraq and is mostly subjected to abiotic stresses such as drought, salinity, pH and temperature. Both alfalfa and its nitrogen fixing symbiotic bacteria *Sinorhizobium meliloti* are affected by these environmental stresses. Enhancing nitrogen fixation biologically could be achieved through selection of tolerant strains of *S. meliloti* to these environmental stresses and inoculating them to the crop, also growing tolerant cultivars. This study examines phenotypic diversity for tolerance to drought, salinity, temperature and pH. Sixty isolates sampled from different areas of Iraq. The results revealed high degree of phenotypic diversity in *Sinorhizobium* populations. Furthermore, the isolates which showed tolerance to drought stress also showed tolerance to salinity and high degree of temperature, indicating direct relationship between three physiological path ways. Also 58.3% of drought tolerant isolates were alkaline tolerant they tolerated up to pH 9, point to say almost all drought tolerant isolates in this study illustrated strong + positive reaction to catalase enzyme. And 91.6% themes were negative for Gelatinase enzyme test. While only 50% of drought sensitive isolates were negative for drought sensitive isolates could grow at high temperature (42 °C).

Key words: *Sinorhizobium meliloti*, phenotypic, abiotic stresses.

1. Introduction

The impact of climate change on biota has recently gained attention, given the significant concerned towards global warming, or local reduction of rainfall in many parts of the world. The resulting land degradation is major constraint of crop yield worldwide, with drought and desertification as important consequences [1]. Furthermore, dry lands cover 40% of the world's land surface and serve as the habitat and surface of livelihood for more than 1 Billion people. Desertification affects 70% of the world's dry lands [2]. Leguminous plants are frequently used for cultivation in degraded soil sites of arid and semi-arid regions because they can grow in barren soils that are unsuitable for most crops [3]. Alfalfa (*Medicago sativa* L.) is a deep-rooted, perennial legume capable of producing high yields of high quality forage. Alfalfa also has the ability to use atmospheric nitrogen (N_2) and deposit significant amounts of N in the soil during growth [4].

The gram-negative bacteria *Sinorhizobium meliloti* is able to interact with the roots of *Medicago* to form nitrogen-fixing nodules and survive as a free living saprophytic bacterium in the soil [5, 6].

In recent years, due to the reduced need for application of nitrogenous fertilizers, the rhizobia have gained a great value and play an important role in improving soil fertility in farming systems [7].

Desiccation negatively affected the relationship

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between *Sinorhizobium* and alfalfa plant by limiting nitrogen fixation [7], therefore, isolation of *Sinorhizobium* strains that are capable of tolerating such stress are essential for efficient nitrogen fixation [8], and enhance production of food and forage in legumes [9]. Also Alikhani [10] concluded that drought tolerance is parallel with salinity tolerance for rhizobia strains. And Mensh [11] said that both acidity and high salinity reduced the growth rate and survival of *Rhizobia* spp.. Keerio [12] found that nitrogen fixation was seriously inhibited at high temperatures.

The aim of the present study was to collect and isolate biodiversity of field population of *Sinorhizobium meliloti* nodulating the common *Medicago sativa* from different geographical areas in Iraq and to characterize their performance against different abiotic stresses including drought, salinity, pH and temperature, so to select tolerant strains which could be used on stressed sites as an inoculum to promote leguminous plant growth in the arid and semi-arid areas.

2. Materials and Methods

This work was carried out at Biology Dept., College of Ibn Al-Haithem, Baghdad University, Iraq, during 2011-2012. The sixty *Sinorhizobium meliloti* isolates used in this study were isolated from nodules sampled from roots of young alfalfa plants grown in different Iraqi areas, using standard procedures [13]. All 60 isolates were gram-negative, fast growing, formed single colonies with diameters of 2-4 mm within 2-3 days on MSY (Mannitol Salt Yeast agar). All *Sinorhizobium* isolates tested for growing on CR (Congo Red) and BTB (Bromothymol Blue) incorporated with MSY agar media, also the isolates tested for PIT (plant infection test) to confirm *Sinorhizobium meliloti* [13]. Purified isolates conserved on agar slant in 4 °C.

2.1 A Biotic Stresses

For studying drought tolerance, the authors used Polyethylene Glycol 6000 (PEG6000, w/v) in MSY broth media at different osmotic pressures ranging from -0.2 to -4 MPa plus the control treatment (no PEG6000), the cultures incubated at 28 °C on a rotary shaker in the dark conditions for 7 days, then bacteria were streaked on MSY agar media plate to assess growth. As for salinity tolerance, this experiment was carried out by growing *Sinorhizobium* isolates on different NaCl concentrations (0.5, 1.5, 2.5, 3.5 and 4.5)% in MSY agar plates plus the control treatment (No NaCl). Isolates incubated at 28 °C for 7 days in dark conditions.

The effect of temperature on *Sinorhizobium meliloti* isolates was performed with different levels of temperatures (4, 28, 37 and 42) °C, the isolates incubated for 7 days in dark conditions. In regards the ability of the isolates to grow in acid and alkaline media, the isolates were inoculated on MSY agar media, pH were adjusted to 4, 6, 8, and 9 by using sterile HCl or NaOH [14], the isolates were kept at 28 °C for 7 days in dark conditions.

2.2 Antibiotic Sensitivity

Resistance to different levels of antibiotic was used to characterize and identify *Sinorhizobium* isolates using Amp, Tetr, Strep and Kar, the concentration is measured as $\mu g \cdot mL^{-1}$.

2.3 Enzymes Test

Catalase test was done according to Mac-Faddin [15], also gelatinase, urease, phenylalanine deaminase and tryptophan deaminase tests were done according to Ronald [16].

2.4 Statistical Analysis

For clustering, the authors used UPGMA algorithm clustering method using past program version 1.92, as for physiological characters data recorded as (+) growth, (-) no growth with three replicate pretreatment.

3. Results and Discussion

3.1 Isolation and Authentication of Sinorhizobium

Sixty isolates of *Sinorhizobium meliloti* were

obtained from root samples of alfalfa plants, grown on MSY+ congo red media within 2-3 days, colonies formed were gram negative, white translucent, glistening, and with diameters of 2-3 mm. These colonies changed the color of media containing BTB to yellow. During sub-culturing, bacterial isolates were purified and refrigerated in 4-5 °C. Plant infection test (PIT) carried out in tubes with proper media for alfalfa legumes in growth chamber.

3.2 Drought Tolerance

This experiment illustrated a significant differences between the isolates, all isolates showed good growth on water potentials -0.1, -0.5 and -1.0 MPa, while in -1.5 MPa water potential, growth of isolates declined to 96.6%, with increasing water potential to -2.5 Mpa, only 50% of isolates grew at this level, finally at -4 MPa water potential, the growth of isolates decline into 6.6% (only four isolates of total sixty), these isolates defined as drought tolerant (Fig. 1).

Drought stress is one of the major environmental factors affecting most crops and decreasing crop yield, the population of soil bacteria decreases along the moisture stress, however, it would not be lost completely and certain soil bacteria can resist these dry conditions, these microorganisms utilize the water pressure in the micro porous of soil and survive by their minimum metabolic activities [10]. The common effect of drought on rhizobia results in osmotic morphology and dehydration of cells [17]. Some authors [18] opined that tolerant rhizobia accumulate osmolyte in response to the osmotic stress, which helps them to overcome effects of osmotic stress due water stresses and since low water potential leads to decrease in water activity, accumulation of salt, an increase in toxic compound which could reach toxic levels cause in depression in viability, when water dips the RNA polymerase ceases to function and metabolism stalls [19]. Also Zahran [7] reported that in osmotic stress a specific protein formed which was detected as new protein band in sodium dodecyl sulfate polyacrylamide gel electrophoresis profile of Rhizobia.

From this experiment, 30 isolates were choosed and divided into the following groups:

(1) 10 sensitive isolates tolerated up to -1.5 MPa water potential, except for isolate Bs 58 was tolerated upto -1.0 MPa;

(2) 8 moderate tolerant isolate tolerated up to -2.5 MPa;

(3) High drought tolerant isolates tolerated from -3 to -4 MPa water potential.

Table 1 showed the geographical origin of the 30 isolates of *Sinorhizobium* which was used in all followed experiments in this study, also Table 2 illustrated the tolerated level of water potential and NaCl for each of the 30 isolates.

3.3 Salinity Tolerance

Symbiotic nitrogen fixation is commonly limited by soil conditions, including salinity, optimization of the benefits of legume inoculation with *Rhizobium* spp. depends on the survival of *Rhizobium* in soil *Sinorhizobium* strains could be used on stressed sites as inoculum to promote leguminous plant growth [20], soil degradation due to stalinization is one of the most serious problems affecting the fertility of soils, especially in arid and semi-arid areas. The results shown in Table 2 demonstrated that among isolates the authors found a high degree of diversity, 26.6% of isolates were highly tolerant to salt concentration from 3.5% to 4.5% NaCl and 50% of these high tolerant to NaCl were also drought tolerant.

Table 2 also showed that 26.6% of isolates were salt sensitive and tolerated up to 1.5% NaCl. Salinity imposes both ionic and osmotic stress. Indeed the regular metabolic processes that are then reflected in protein profiles [21]. A significant positive correlation was found between the salt tolerance and adaptation of Rhizobial strains in drought conditions, this result agreed with Abolhasani [20] and Abdel-Salam [22]. Miller [23] indicated that genes involved in

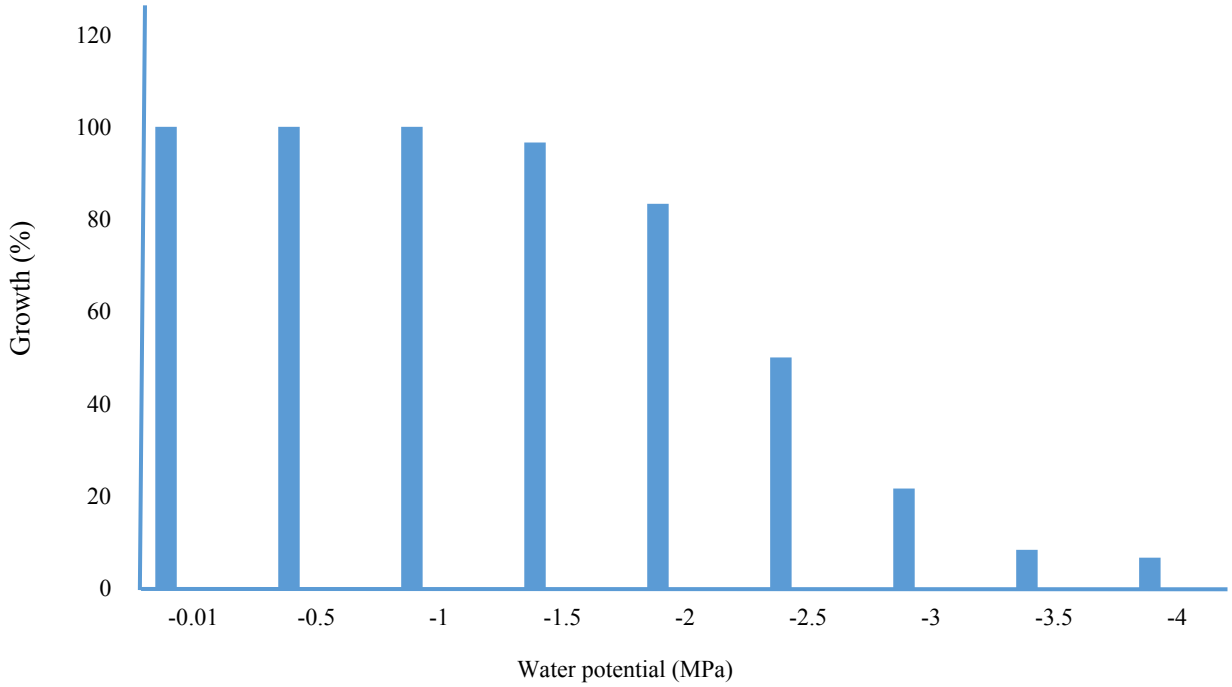

Fig. 1 Growth of *Sinorhizobium* isolates under different water potential levels.

Table 1 Location and nomination of *Sinorhizobium meliloti* isolates divided into three groups based to their drought tolerance.

Group 1: Tolerant		Group 2: Sensitive		Group 3: Moderate	
Isolate No.	Geographical origin	Isolate No.	Geographical origin	Isolate No.	Geographical origin
Bs 12	Amryea faluja 2. Iraq	Bs 15	Seqlawea 3, Iraq	Bs 2	Khanqeen 2, Iraq
Bs 23	Abu-Ghreb, dr. Harb	Bs 25	Babel-kefl 2, Iraq	Bs 7	Tarmea 2, Iraq
Bs 29	Ramady 3, Iraq	Bs 26	Babel-kefl 3, Iraq	Bs 13	Seqlawea 1, Iraq
Bs 30	Ramady 4, Iraq	Bs 42	Kufa-zerga 7, Iraq	Bs 16	Seqlawea 4, Iraq
Bs 32	Abu-Ghareb 2, Iraq	Bs 43	Kufa-zerga 8, Iraq	Bs 31	Abu-Ghareb 1, Iraq
Bs 38	Nasryea, Iraq	Bs 44	Kufa-zerga 9, Iraq	Bs 46	Kufa-Abasyea 2, Iraq
Bs 40	Balad 2, Iraq	Bs 54	Kufa-Qzwenea 4, Iraq	Bs 48	Nasryea_Masiab 2, Iraq
Bs 41	Balad 3, Iraq	Bs 55	Mahawel 1, Iraq	Bs 49	Nasryea_Masiab 3, Iraq
Bs 50	Amryea faluja 3, Iraq	Bs 57	Mahawel 3, Iraq		
Bs 53	Baquba 3, Iraq	Bs 58	Saydea, Baghdad, Iraq		
Bs 59	Ramady 4, Iraq				
Bs 60	Seqlawea 7, Iraq				

tricarboxylic acid, in the uptake of carbon source, in respiratory chain and ribosomal genes were repressed under salt stress.

3.4 pH Tolerance

Sinorhizobium isolates (in general) showed well adapted to high and low pH. 40% of the isolates grew at pH 9 while 16.6% grew at pH 4, moreover all *Sinorhizobium* isolates grew well at pH 6, also results showed that 58.3% of isolates which grew in alkaline conditions (pH 9) were drought tolerant (tolerated low water potential from -3 to -4 MPa) (Table 3).

The results agreed with Abolhasani [20] that all strains tolerating salt concentration from 2.5% to 4.5% NaCl were highly resistance to alkaline conditions, also data in Table 3 consent with Ali [24] reported that a few of the Rhizobial isolates from Rajasthan, a dry region in India, were able to grow at pH 4.5, moreover,

Table 2 Drought and salinity tolerating levels of *Sinorhizobium* isolates.

Isolate No.	Highest water potential tolerated (MPa)	Highest NaCl con. tolerated (%)	Isolate No.	Highest water potential tolerated (MPa)	Highest NaCl con. tolerated (%)
Bs 12	-4	4.5	Bs 42	-1.5	2.5
Bs 23	-3	3.5	Bs 43	-1.5	2.5
Bs 29	-3	2.5	Bs 44	-1.5	1.5
Bs 30	-3.5	4.5	Bs 54	-1.5	1.5
Bs 32	-4	2.5	Bs 55	-1.5	1.5
Bs 38	-4	4.5	Bs 57	-1.5	1.5
Bs 40	-3.5	4.5	Bs 58	-1	1.5
Bs 41	-4	4.5	Bs 2	-2.5	2.5
Bs 50	-3	2.5	Bs 7	-2.5	4.5
Bs 53	-3	2.5	Bs 13	-2.5	2.5
Bs 59	-3	2.5	Bs 16	-2.5	4.5
Bs 60	-3	2.5	Bs 31	-2.5	4.5
Bs 15	-1.5	1.5	Bs 46	-2.5	2.5
Bs 25	-1.5	1.5	Bs 48	-2.5	2.5
Bs 26	-1.5	1.5	Bs 49	-2.5	2.5

Table 3 *Sinorhizobium* isolates grown on different levels of pH.

Isolate No.	pH levels				Isolate No.	pH levels			
	4	6	8	9		4	6	8	9
Bs 12	+	+	+	+	Bs 42	-	+	+	+
Bs 30	+	+	+	+	Bs 43	+	+	+	+
Bs 23	-	+	+	-	Bs 44	-	+	+	-
Bs 29	-	+	+	-	Bs 54	-	+	+	-
Bs 32	+	+	+	+	Bs 55	-	+	+	-
Bs 38	-	+	+	+	Bs 57	-	+	+	+
Bs 40	-	+	+	-	Bs 58	-	+	+	-
Bs 41	-	+	+	-	Bs 2	-	+	+	-
Bs 50	-	+	+	+	Bs 7	-	+	+	-
Bs 53	-	+	-	-	Bs 13	-	+	+	-
Bs 59	-	+	+	+	Bs 16	-	+	+	+
Bs 60	-	+	+	+	Bs 31	+	+	+	-
Bs 15	-	+	+	-	Bs 46	-	+	+	+
Bs 25	-	+	+	-	Bs 48	-	+	-	-
Bs 26	-	+	+	-	Bs 49	-	+	+	-

-: No growth; +: Growth.

Thqmi-Alami [21] confirmed that *Sinorhizobium* nodulating alfalfa plant were acid sensitive.

3.5 Temperature Tolerance

Generally all isolates exhibited good growth at 28 °C and most of them also showed good growth at 37 °C except for Bs 57 didn't grew at this temperature. Nevertheless at 4 °C only five isolates could live at this temperature (Table 4).

Some researches confirmed these results [21, 24] that optimum temperature for growth of root nodulating bacteria ranged from 25-31 °C. Also Ihsan [25] reported that it is rare to Rhizobia to grow at 4 °C. The ability of Rhizobia to with stand high temperature dose not linked to their ability to survive desiccation [26]. Thus, identification of heat-tolerant strains may

Table 4 ***Sinorhizobium*** **isolates growth on different levels of temperature (°C).**

Isolate No.	Temperature (°C)				Isolate No.	Temperature (°C)			
	4	28	37	42		4	28	37	42
Bs 12	-	++	+	+	Bs 42	-	++	+	-
Bs 30	+	++	++	++	Bs 43	-	++	+	-
Bs 23	-	++	++	-	Bs 44	-	++	+	-
Bs 29	-	++	+	-	Bs 54	+	++	+	+
Bs 32	-	++	++	+	Bs 55	-	++	+	-
Bs 38	-	++	+	-	Bs 57	-	++	-	-
Bs 40	-	++	++	+	Bs 58	-	++	+	-
Bs 41	+	++	+	+	Bs 2	-	++	++	+
Bs 50	-	++	+	-	Bs 7	-	++	++	-
Bs 53	-	++	+	-	Bs 13	-	++	+	+
Bs 59	-	++	+	+	Bs 16	+	++	+	+
Bs 60	-	++	+	+	Bs 31	-	++	+	-
Bs 15	-	++	+	+	Bs 46	+	++	++	-
Bs 25	-	++	+	-	Bs 48	-	++	++	+
Bs 26	-	++	+	-	Bs 49	-	++	+	-

-: No growth; +: Weak growth; ++: Good growth.

not enhance survival during desiccation, unless temperature, rather than drought, is the selection stress [26]. In regards to this study, the increase in temperature led to noticeable decline in growth. At 42 °C only one isolate give good growth (Bs 30), while the other showed weak/no growth, data in Table 4 revealed that 43.3% of *Sinorhizobium* isolates could grow at 42 °C this result consent with Ihsan who [25] reported that only *Sinorhizobium meliloti* isolates could grow at 42 °C temperature level. Data also revealed that 58.3% of drought tolerant isolates in this study were also high temperature tolerant (grow at 42 °C), which 20% of drought sensitive isolates were able to with stand and grow weakly at 42 °C.

3.6 Antibiotic Sensitivity

All 30 isolates were tested against four kinds of antibiotics in 10, 25 and 50 $\mu g \cdot mL^{-1}$ concentration. The results revealed that all isolates of *Sinorhizobium* were highly resistance to Erythromycin in all it concentration levels, and 43% of isolates could resist Kanamycin in 10 $\mu g \cdot mL^{-1}$ (Table 5).

3.7 Enzymes Test

The results revealed that all isolates gave positive reaction to catalase except of two isolate Bs 43 and Bs 46 were negative to the test. As clearly shown in Table 6 that 83.3% of drought tolerant isolate gave a strong positive reaction to this enzyme, this result consent with Haythem [27] indicated that NaCl and PEG tolerant strains showed increase in catalase production under water deficient.

In regards to Gelatinase data showed 91.6% of drought tolerant isolate were negative for Gelatinase test. While 50% of drought sensitive isolate were negative for the same enzyme test. As for Urease Table 6 showed 83.3% of drought tolerant isolates gave negative reaction for Urease compared to 20% of drought sensitive gave negative reaction for Urease.

3.8 Cluster Analysis

The results in Fig. 2 represent a dendrogram obtained by UPGMA cluster using all data from the physiological characters experiments. The authors chose 11 isolates, four were drought sensitive (Bs 44, 51, 55, and 58), four drought tolerant (Bs 12, 30, 38 and 41) and three drought moderate tolerant (Bs 49, 31 and 16). The cluster analysis (similarity level of

Table 5 Antibiotic resistance of thirty *Sinorhizobium* isolates at different concentration of antibiotics.

Antibiotic	Resistance of isolates, %		
	10 μg·mL^{-1}	25 μg·mL^{-1}	50 μg·mL^{-1}
Erythromycin	93	93	90
Streptomycin	66	53	16
Kanamycin	43	30	20
Ampicilin	83	36	26

Table 6 Enzymes reaction pattern of *Sinorhizobium* isolates.

Isolate No.	Catalase	Urease	Phenylalanine deaminase	Tryptophan deaminase	Gelatinase
Bs 12	++	-	-	-	-
Bs 30	++	-	-	-	-
Bs 23	++	+	+	+	-
Bs 29	++	-	-	-	-
Bs 32	++	-	+	-	-
Bs 38	++	-	-	-	-
Bs 40	++	+	-	-	-
Bs 41	++	-	-	-	-
Bs 50	++	-	+	-	-
Bs 53	+	-	-	+	+
Bs 59	++	-	-	-	-
Bs 60	++	-	-	-	-
Bs 15	++	+	+	-	+
Bs 25	+	+	-	+	-
Bs 26	++	+	+	-	-
Bs 42	+	+	-	+	-
Bs 43	-	-	-	-	+
Bs 44	+	+	+	+	+
Bs 54	+	+	+	-	+
Bs 55	+	+	+	+	-
Bs 57	+	-	-	-	-
Bs 58	+	+	-	+	+
Bs 2	+	-	-	-	+
Bs 7	++	+	+	+	+
Bs 13	+	-	-	+	-
Bs 16	++	-	-	-	-
Bs 31	++	-	-	-	-
Bs 46	-	+	-	+	+
Bs 48	+	+	+	-	+
Bs 49	++	-	-	-	-

Catalase: -: No reaction; +: Weak reaction; ++: Good reaction.
Other enzyme: -: negative; +: positive.

100) showed that all eleven isolates divided into two major clusters with 49% similarity, first major cluster included all four drought sensitive which they were also salt sensitive and abled to grow at 28-37 °C, except for isolate Bs 54 was abled to grow at 4-42 °C (which placed it in subgroup with similarity of 77% to the other three drought sensitive tolerant isolates, regarding growing on acidity/alkaline conditions, all isolates in the first major group were abled to grow at pH 6 and 8 only.

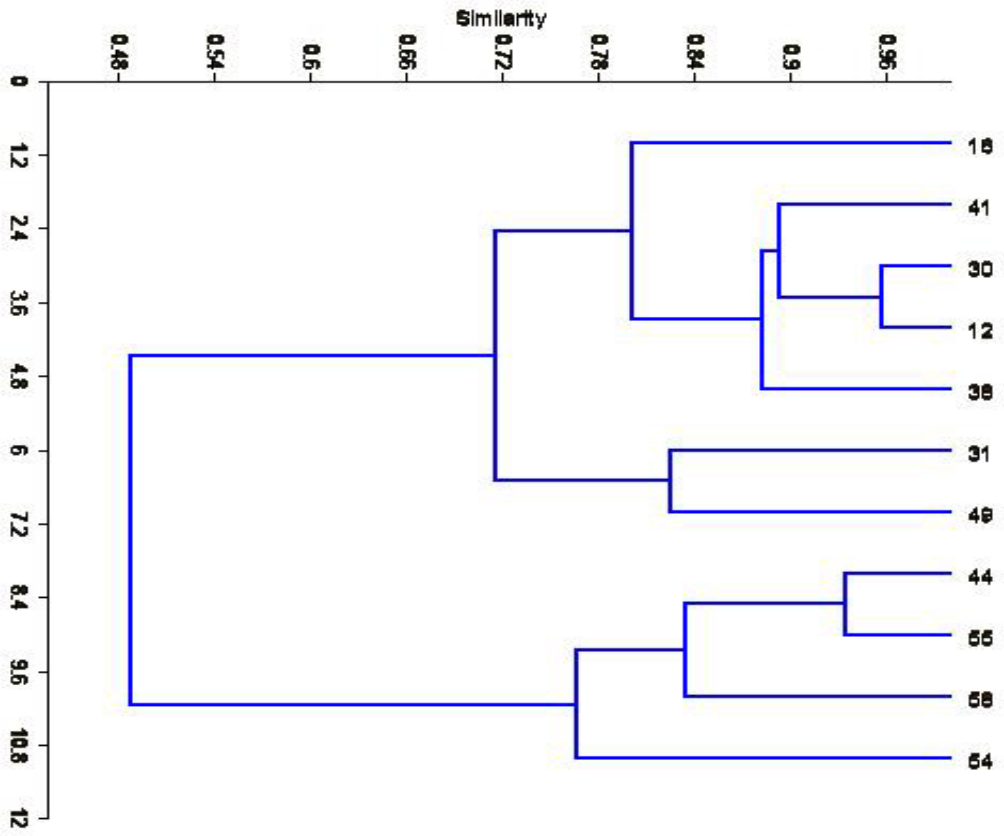

Fig. 2 Dendrogram showing the relationship among *Sinorhizobium meliloti* based on phenotypic variation.

The second major group divided into two subgroup with 71.7% similarity, first subgroup included two moderate drought tolerant isolates (also they were salt tolerant) and they were abled to grow on alkaline conditions. The second subgroup included all drought tolerant isolates plus Bs 16 (drought moderate tolerant isolate) with 80% similarity to the other isolate in the second subgroup.

All the isolates in the second subgroup were salt tolerant and abled to grow at 4-42 °C, except for Bs 38 was abled to grow at 28-37 °C.

4. Conclusions

Results reported here showed varied degrees of drought and other a biotic stresses tolerance under laboratory conditions. Also it can be concluded that drought tolerance is parallel with salinity tolerance for *Sinorhizobium* strains. Further experiments in the field are required to correlate these results. The resistance patterns found among the indigenous *Sinorhizobium* isolates are reflecting the environmental stresses pressure predominant in their locations and are very good examples of the importance of using efficient indigenous strains for plant inoculation in each specific area. We hoped more supplementary investigations would be conducted to introduce superior strains to produce commercial biofertilizers and enhance production of food and forage legumes in semi-arid and arid regions of the world.

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Study of the Potential Value of *Ilex affinis* (Aquifoliaceae) as a Novel Source for the Food and Pharmaceutical Industries

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Abstract: *Ilex paraguariensis* St. Hilaire (Aquifoliaceae) is processed industrially to produce the commercial product "yerba mate" which is used as a tea-like beverage. It is one of the most commercialized plants of South America. It is exported to the US, Europe and Asia as vegetal drug or extracts used in complementary and alternative medicine and in formulations for functional foods due to its properties as a CNS stimulant, diuretic, weight reducing, antioxidant and antihypercholesterolemic, among others. *Ilex affinis* grows in the same habitat and is used as substitute or adulterant of *I. paraguariensis*. This species was never investigated before. The objective of this work was to assess the phytochemical composition and to determine the pharmacological activity, according with the major compounds present in it. The results showed small quantities of caffeine and theobromine, but a considerable amount of polyphenols, especially chlorogenic acid and isochlorogenic acid. *I. affinis* extracts presented scavenging activity on free radical DPPH in a concentration-dependent manner. Antiproliferative action on lymphoma cell line exerting both cytostatic and cytotoxic activities was also demonstrated.

Key words: *Ilex affinis*, *Ilex paraguariensis*, polyphenols, chlorogenic acid, antiproliferative activity.

1. Introduction

Ilex paraguariensis St. Hilaire (Aquifoliaceae) is a plant which grows naturally in NE Argentina, SE Brazil, E Paraguay and Uruguay and it is cultivated in the first three countries [1]. This species is industrially processed to produce the commercial product "yerba mate", used as a tea-like beverage [2]. It is one of the most commercialized plants of South America where approximately 30% population drink more that 1 L/day of this beverage [3]. It is exported to the US, Europe and Asia as vegetal drug or extracts used in complementary and alternative medicine and in formulations for functional foods due to its properties as a CNS stimulant, diuretic, weight reducing, antioxidant and antihypercholesterolemic, among others [4].

Some related species of the genus *Ilex* from the same habitat, are used as substitutes or adulterants of *I. paraguariensis*. They are: *I. affinis*, *I. dumosa*, *I. brevicuspis* and *I. brasiliensis*, among others [5]. *I. affinis* has never been reported in Argentina. Collection campaigns conducted recently in the province of Misiones allowed to find this species in our country [6]. According to our knowledge, no phytochemical or pharmacological researches on this species were performed previously.

The presence of caffeoyl derivative compounds (caffeic acid, chlorogenic and isochlorogenic acids) in *I. paraguariensis*, *I. dumosa*, *I. brevicuspis* and *I. brasiliensis* has been reported [7-9]. *I. paraguariensis* also contain considerable amounts of methyl xanthines [2].

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Nowadays, *I. dumosa* is cultivated and it has recently been included in the Argentina Food Code, as an herb for infusions, to be used mixed with *I. paraguariensis* [10].

I. brevicuspis showed antioxidant, choleretic and intestinal propulsion activity in rats [7]. In previous works, we demonstrated that *I. brasiliensis* exerted antiproliferative and apoptosis activity on a lymphoma cell line and chlorogenic acid proved to be one of the compounds associated to this activity [9].

There is an increasing interest in the antioxidant effects of compounds derived from herbs that could be relevant in relation to their nutritional effects and their role in health diseases.

Wine is one of the most important social beverages among Europeans. Many studies showed the cardioprotective properties of red wine. Resveratrol has been identified as one of the more powerful bioactives, but many other compounds including cinnamic acid derivatives, tannins and other polyphenols have been related to its beneficial effects [11, 12].

Low incidence of diabetes was linked to consumers of products containing chlorogenic acids. Green coffee beans may contain up to 55% of chlorogenic acids [13].

In a previous work, *L. paraguariensis* showed 2-times higher antioxidant activity compared to red wine [14]. The antioxidant properties of *I. paraguariensis* have been linked to the health benefits of yerba mate.

Taking into account the results of our previous investigations on *Ilex* spp., the objective of this work was to assess the phytochemical composition of *I. affinis* and to determine the pharmacological activity, according with the major compounds present in it.

2. Material and Methods

2.1 Plant Material

I. affinis was collected in San Ignacio, province of Misiones, Argentina and a sample was taken from an abundant specimen—leg. Keller & Keller 9588—kept at the herbarium BAF.

2.2 Preparation of Plant Extracts

Dried leaves were ground to fine powder. Decoctions extracts were prepared in order to compare with preparations commonly used by local people. One gram was boiled with 10 mL of water during 20 min, then it was cooled to 40-45 °C, filtered and the volume adjusted to 5 mL.

2.3 Determination of Caffeoyl Derivative Compounds, Flavonoids and Methylxanthines by HPLC

Previously validated methods were used for the analysis of caffeoyl derivative compounds, flavonoids and methylxanthines [2, 7]. A reverse phase column applying two different gradients, using as the mobile phase: Solvent A: water:acetic acid (98:2); solvent B: methanol:acetic acid (98:2). For the analysis of caffeoyl derivative compounds, the gradient used was: 15% B to 40% B, 30 min; 40% B to 75% B, 10 min; 75%B to 85% B, 5 min. Flow rate: 1.2 mL/min. For the analysis of methylxanthines the gradient used was: 17% B to 20% B, 10 min; 20% B isocratically, 5 min; 20% B to 23% B, 10 min; 23% B to 100% B, 5 min. Flow rate: 1.0 mL/min. The separation column was IB-SIL RP 18 (5 μm, 250 × 4.6 mm I.D.) Luna. Detection was carried out by UV Varian 9050 UV Detector and Varian 9065 Photodiode-Array Detector. UV: 325 nm (caffeoyl derivatives); 255 nm (flavonoids); 273 nm (methylxanthines). The equipment had a Rheodyne injector, fitted with a 100 μL loop. Quantification was achieved by the external standard method using standards compounds (Sigma-Aldrich Argentina, Buenos Aires) The amount of 3,4-dicaffeoylquinic, 3,5-dicaffeoylquinic and 4,5-dicaffeoylquinic isomer acids were calculated and expressed as cynarin (1,5-dicaffeoylquinic acid).

2.4 Determination of the Free Radical Scavenging Activity by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) Free-Radical Scavenging Assay

Scavenging activities of *I. affinis* extracts on the stable free radical DPPH were assayed using the

modified Bloi's method in which the bleaching rate of DPPH is monitored at a characteristic wavelength in presence of the sample [15]. Briefly a volume of 0.1 mL of an aqueous dilution of the extracts were mixed with 0.5 mL of a 500 M DPPH solution in absolute ethanol and 0.4 mL of a 0.1 M Tris-ClH buffer pH 7.4. The mixture was kept for 20 min in the darkness and then the absorbance was read at 517 nm. The percentage of decrease of DPPH bleaching was calculated by measuring the absorbance of the sample and applying the following equation:

$$\text{Inhibition (\%)} = [1 - (As/Ao)] \times 100$$

where: *As* is absorbance of sample (*I. affinis* extracts) and *Ao* is the absorbance of the DPPH solution. A standard ascorbic acid solution 100 ug/mL was used as positive control for antioxidant activity.

2.5 Proliferation, Viability and Apoptosis Assays

A tumoral cell line called EL4 was used. EL4 cells (ATCC) are a T cell lymphoma induced in a C57BL mouse by 9,10-dimethyl-1,2-benzanthracene. The cells were cultured at optimal concentrations in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 2 mM glutamine and antibiotics: 100 U/mL penicillin and 100 μg/mL streptomycin. The effect on proliferation was evaluated by the MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-di-phenyl tetrazolium bromide (Sigma, Buenos Aires, Argentina) method. Cells were incubated alone or in presence of different concentrations of *I. affinis* extracts (from 0.01 to 1,000 μg/mL) for 24 h. Then, MTT was added and the purple formazan formed was solubilized by addition of acidic iso-propanol. The absorbance was measured at 570 nm and results were expressed as percentage of proliferation. The same method was employed to determine cell viability and in this case results were expressed as percentage of viability relative to control [16].

2.6 Statistical Analysis

Data were expressed as means ± SD or SEM of two or three independent experiments carried out in duplicate. A one-way ANOVA with a posteriori the Dunnett's test were used to evaluate the significancy of results. A probability (P) value < 0.01 was considered significant [17].

3. Results and Discussion

The identification of compounds present in the extracts was carried out using validated HPLC methods and determined by the coincidence of their retention times with those of reference compounds and the UV/Vis spectra provided by the DAD detector.

The phytochemical study showed traces of methylxanthines (caffeine and theobromine) and a considerable amount of polyphenols. The following compounds were isolated and quantified and the results are expressed as % on dried weight: a) caffeoyl derivative compounds (chlorogenic acid: 0.0398 ± 0.0004; 3,4-dicaffeoylquinic acid: 0.0166 ± 0.0001; 3,5-dicaffeoylquinic acid: 0.0296 ± 0.0003 and 4,5-dicaffeoylquinic acid: 0.0392 ± 0.0004); b) flavonoids (rutin: 0.134 ± 0.001 and quercetin: 0.0066 ± 0.0001). Kaempferol was not detected. Detection limit: 0.2 ppm. Quantification limit: 1.0 ppm.

There is evidence that plant-derived compounds may have beneficial effects on human health and that some of them (caffeoyl derivatives and flavonoids) exert antioxidant activity [18, 19].

In this study, *I. affinis* extracts presented scavenging activity on free radical DPPH in a concentration-dependent manner. The concentration of 100 μg/mL exerted an scavenging effect similar to that exerted by the antioxidant control ascorbic acid (100 μg/mL) (Fig. 1).

I. affinis showed antiproliferative activity on a lymphoma cell line. One drug can decrease cell proliferation by a cytostatic or a cytotoxic action. Cytostatic effect is exerted when a drug decreases cell proliferation, but does not modify cell viability; really, the decrease in cell proliferation is not due to the

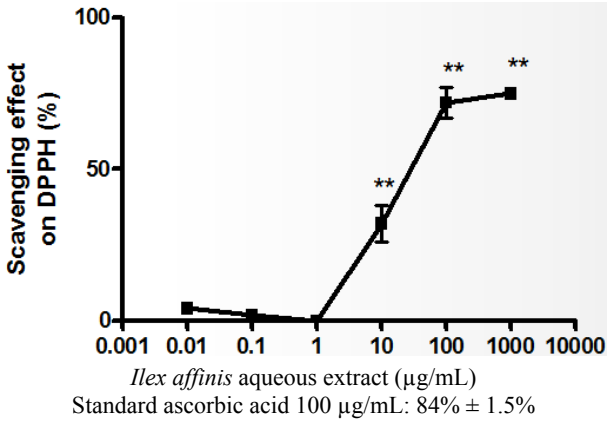

Fig. 1 Effect of different concentrations of *I. affinis* on free radical (DPPH) elimination. Results represent mean ± SEM of two experiments made by duplicate. ** $P < 0.01$ significantly differences between control (DPPH alone) and treatments accordingly with ANOVA + Dunnett's test.

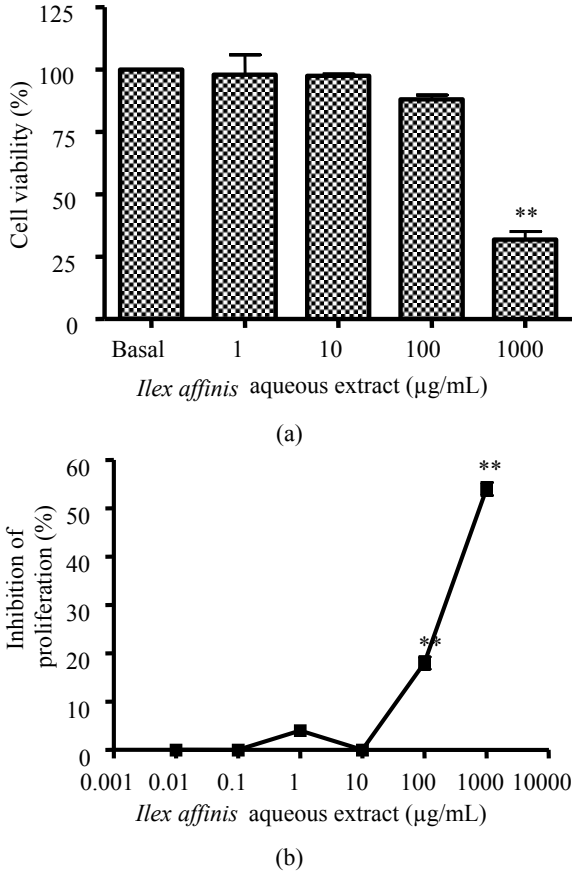

Fig. 2 Effect of different concentrations of *I. affinis* on lymphoma cell proliferation (A) and on cell viability (B). Cells were incubated during 24 h with the aqueous extract from 0.01 to 1,000 µg/mL. Results represent mean ± SEM of three experiments made by triplicate. ** $P < 0.01$ significantly differences between control and treatments in accord with ANOVA + Dunnett's test.

increase of cell mortality. Cytotoxic effect is exerted when a drug decreases cell proliferation, but a decrease in cell viability is also observed, so the drug decreases cell proliferation because it is killing the cells.

The activity displayed by *I. affinis* occurred by both, cytostatic and cytotoxic effects as it is shown in Fig. 2. The effect depended on the analyzed concentrations following a concentration-response relation-ship.

Antiproliferative action has been observed in other *Ilex* species, e.g., an *I. paraguariensis* extract was shown to exert an antiproliferative effect on an oral carcinoma by inhibition of topoisomerase II [20]; this specie was also reported to inhibit the growth of ras-transformed endothelial cells [21]. *I. brasiliensis* also exerted antiproliferative and apoptosis activity on a lymphoma cell line [9].

4. Conclusions

I. affinis aqueous extracts showed considerable amounts of polyphenols, especially chlorogenic acid and isochlorogenic acid and exerted antioxidant activity. Antiproliferative action on lymphoma cell line exerting both cytostatic and cytotoxic activities was also demonstrated. The results obtained in this work suggest the potential value of *I. affinis* for the development of novel products in the food and pharmaceutical industries.

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The Effect of Age and Markers of Obesity on Bone Density in Iraqi Postmenopausal Women

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Abstract: Osteoporosis is a disease characterized by a low bone mineral density and structural deterioration of bone tissue. Leptin is considered to play a role in the maintenance of energy balance and body weight. Weight and body mass index are associated with low bone mineral density with high serum leptin concentration in older women. The study is designed to elucidate the impact of age and BMI (Body Mass Index) on osteoporotic patients (Iraqi postmenopausal women) and the functional role of leptin in those patients. A total of 72 patients divided into three groups according to BMI and two groups according to age: BMD (bone mineral density), *T*-score and serum leptin concentration increased as BMI increased, while with increased age, BMD and *T*-score deceased and serum leptin concentration increased. There is a significant difference of BMD and *T*-score in BMI (25-30) group and BMI (> 30) group from that in BMI (< 25) group. In conclusions: Both age and BMI have an impact on osteoporosis although age shows more impact on the severity of the disease than does BMI. Studying the direct impact of leptin on BMD may open the way in using new methods in treating and preventing the osteoporosis in patients with risk factors.

Key words: Osteoporosis, leptin, obesity, BMD, BMI.

1. Introduction

Osteoporosis is a chronic, and a progressive disease of multifactorial etiology which has been most frequently recognized in elderly white women, although it does occur in both sexes, all races and all age groups. It is a systemic skeletal disease characterized by low bone mass and micro-architectural deterioration of bone tissue, with a consequent increase in bone fragility [1].

Despite the adverse effects of osteoporosis, it is a condition that is often overlooked and undertreated, because in large part it is often clinically silent before manifesting in the form of a fracture. Failure to identify at risk patients, to educate them, and to implement preventive measures may lead to tragic consequences [2].

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BMD (bone mineral density) in a patient is related to the peak bone mass and, subsequently, bone loss. Whereas the *T*-score is the patient's bone density compared with the BMD of control subjects who are at their peak BMD, the Z-score reflects a bone density compared with that of patients matched for age and sex [3-6].

The World Health Organization's (WHO) definitions of osteoporosis based on BMD measurements in white women are summarized in Table 1.

Risk for osteoporosis increased with age as BMD declined. Senile osteoporosis is most common in persons aged 70 years or older. Secondary osteoporosis, however, can occur in persons of any age. Although bone loss in women begins slowly, it speeds up around the time of menopause, typically at about or after age 50 years. The frequency of postmenopausal osteoporosis is highest in women aged 50-70 years [7-9].

Table 1 WHO definition of osteoporosis based on BMD measurements by DXA [5, 6].

Definition	Bone mass density measurement	*T*-Score
Normal	BMD within 1 SD of the mean bone density for young adult women	≥ −1
Low bone mass (osteopenia)	BMD 1-2.5 SD below the mean for young-adult women	between –1 and –2.5
Osteoporosis	BMD ≥2.5 SD below the normal mean for young-adult women	≤ –2.5
Severe or "established" osteoporosis	BMD ≥2.5 SD below the normal mean for young-adult women in a patient who has already experienced ≥1 fractures	≤ –2.5 (with fragility fractures)

Age-specific variations in bone density are mainly determined by genetic factors, but it also may due to estrogen deficiency and vitamin D deficiency that is related to age. Most investigators registered a decrease in the osteoprogenitor pool of bone marrow with advancing age [10]. Lack of exercise, prolonged immobilization from injuries, medications and underlying disease states may contribute with increase age [11, 12].

Leptin is produced primarily in fat cells, large fat cells produce more leptin than small ones and serum leptin concentrations are highly correlated with body fat content. Leptin mRNA and secretion by adipocytes declines rapidly during starvation. These processes are stimulated by insulin, glucocorticoids and tumor necrosis factor-alpha [13, 14].

This study is designed to elucidate the impact of age and BMI on osteoporotic patients (Iraqi postmenopausal women) and the functional relationship of leptin in these patients.

2. Subjects and Methods

This study was conducted in Baghdad Teaching Hospital (Rheumatology Clinic and Teaching Laboratories) during the period of Jan.-July 2012.

Seventy-two postmenopausal women (45-75 years) were identified as newly primary osteoporosis by clinical examination and radiological images (DXA) according to WHO criteria [5, 6]. Patients with secondary osteoporosis were excluded.

2.1 Measurement of BMD by DXA (Dual-Energy X-Ray Absorptiometry)

BMD was measured at the lumbar spine (L1-L4), using dual-energy x-ray absorptiometry (DXA) machine (Lunar system, Dexxum). BMD was expressed as *T*-score considering the diagnostic criteria for osteoporosis established by World Health Organization (WHO) [5, 6].

Fig. 1 is DXA report for one of osteoporotic patients in the study, from BMI < 25 group and age group (61-75 years) with: BMD = 0.558 gm/cm^2 and *T*-score = −4.2 at L1.

2.2 Blood Samples Collection

Three milliliters of venous blood were aspirated using disposal syringes. Samples were collected between (09.00 am-12.00 pm). The blood was allowed to clot in plain tubes for (30 to 45) min at room temperature and the serum recovered by centrifugation at (3,000 rpm) for (10 min) and transferred into plain plastic tubes and kept frozen at (-20 °C) until time of assay.

2.3 Serum Leptin Assay

Assay was carried out by ELISA technique and the normal values according to the kit used were:

Females 7.36 ± 3.73 ng/mL

Male 3.84 ± 1.79 ng/mL

The kit used was from DRG instruments GmbH, Germany.

Descriptive analysis is used to show the mean, standard deviation for BMI, age, leptin, BMD and *T*-score.

Count and frequency is used to describe the different groups according to BMI group. Chi-square is used to show the relation between BMI groups with age groups.

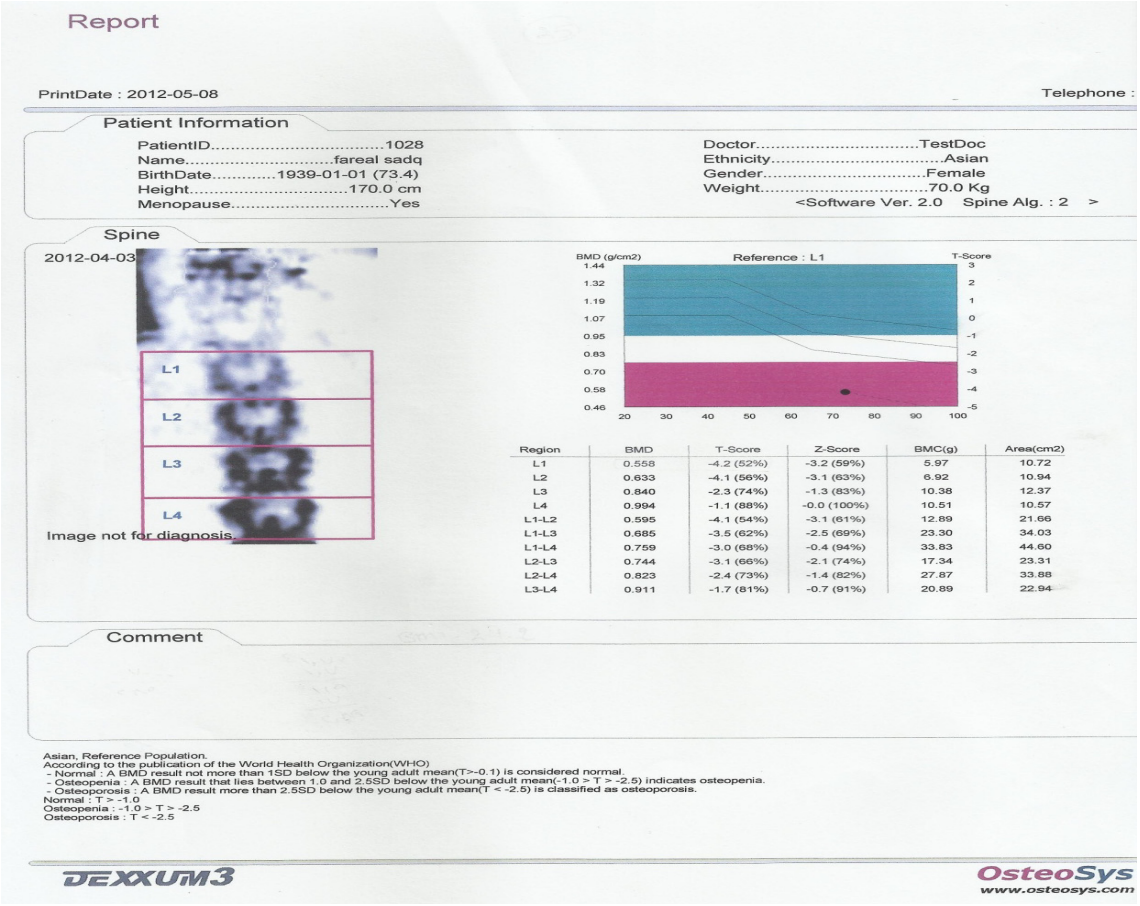

Fig. 1 DXA report.

ANOVA was used to study the differences in groups for BMI, age, leptin, BMD and *T*-score. *P* value of less than 0.05 was considered significant. SPSS (Statistical Package for Social Sciences) version 17 was used for analysis.

3. Results and Discussion

The results showed that both age and BMI have an impact on the severity of osteoporosis. So understanding the impact of BMI and age on osteoporotic patients could help in preventing and early diagnosis of this disease, which has tragic consequences.

The severity of osteoporosis (BMD and *T*-score) declines as BMI increases from lean weight (BMI < 25) to over weight (BMI 25-30) to obese patients (BMI > 30) who have the highest BMD and *T*-score.

Serum leptin concentration (ng/mL) increased with BMI, from lean to over-weight and reached its highest concentration in obese patients. BMD (gm/cm^2) and *T*-score also increased with the increase in BMI. There is a significant difference of BMD and *T*-score in group BMI (25-30) and group BMI > 30 form that in group BMI < 25, while there is no significant difference between BMD and *T*-score in the groups of BMI (25-30 and > 30). Leptin does not show any significant difference between the different BMI groups, as shown in Table 2.

Serum leptin concentration (ng/mL) increased with age, while BMD and *T*-score decreased with age, is shown in Table 3.

These results agree with Morin, et al. who concluded that low weight and BMI predict osteoporosis and are associated with increased fracture risk in younger women [15].

Also our results agrees with that of Jonathan P

Table 2 Mean ± SD and multiple comparisons of studied parameters in different BMI groups.

Parameters	BMI < 25 22.59 ± 1.47	BMI 25-30 28.32 ± 1.14	BMI > 30 34.39 ± 2.9
Leptin (ng/mL)	19.84 ± 15.49	21.19 ± 17.66	27.68 ± 16.15
BMD (gm/cm^2)	0.639 ± 0.92	0.693 ± 0.686 a	0.725 ± 0.044 a
T-score	−3.72 ± 0.737	−3.22 ± 0.501 a	−2.98 ± 0.430 a

a: significant difference from (BMI < 25) group.

Table 3 Mean ± SD of studied parameters in different age groups.

Parameters	Age (45-60 year) 54.7 ± 4.16	Age (61-75 year) 66.94 ± 4.22
Leptin (ng/mL)	20.31 ± 14.01	24.83 ± 18.51
BMD (gm/cm²)	0.73 ± 0.03	0.63 ± 0.07
T-score	−2.97 ± 0.16	−3.78 ± 0.52

Castro, et al. who found that with each unit increase in BMI, BMD increases for white women [16].

Ashish Atreja, et al. showed that majority of patients with osteoporosis (83.3%) missed by the current guidelines had low BMI as we have proved also. Multivariate logistic regression analysis showed that low BMI was the strongest risk factor for osteoporosis [16, 17].

As BMI increase, it is considered as weight bearing exercise. Mechanical loads applied to bone are thought to be communicated through the bone by way of a mechanical signal detected by either bone lining cells or osteocytes or both. It is believed that these mechanical signals lead to the generation of chemical signals involved in the regulation of bone formation and remodeling. The osteocytes, in particular, have received much attention in this regard. Osteocytes are connected to each other and to osteoblasts by way of cellular processes within canaliculi and are linked by gap junctions. This network allows for the possibility of electrical coupling as well as intracellular and extracellular molecular transport in cells deep within bone tissue [18].

Serum leptin concentration increases with BMI from lean weight (BMI < 25) to over weight (BMI 25-30) till it reaches the highest serum leptin concentration in obese patients (BMI > 30).

Serum leptin concentration (ng/mL) increased as BMI increased, the highest concentration of serum leptin was in BMI > 30.

On the other hand serum leptin concentration (ng/mL) increased with age, as age group (61-75 year) had higher serum leptin concentration than that of age group (45-60 year). These findings agrees with Masoud Y Al Maskari, et al. who concluded that serum leptin levels are higher in the Omani obese group and correlate positively with body fatness and obesity [18].

Although deficiency of leptin [19] or the leptin receptor [20] resulted in obesity, most human obesity is associated with elevated leptin levels [21]. Leptin mRNA levels and secretion during a 2-h *in vitro* incubation correlate with obesity (as assessed by BMI or body fat) and even more tightly with fat cell size [22, 23]. The increased leptin secretion and synthesis in obese fat cells could simply be secondary to increased leptin mRNA levels. However, a number of lines of evidence [24] and other studies in human and rat adipose tissue suggest that leptin production is also regulated at posttranscriptional steps, including translation and secretion [25, 26].

BMD (gm/cm²) increased as the BMI increased, the lowest BMD was in BMI < 25 group and the highest one was in BMI > 30 group.

BMD (gm/cm^2) decreased when the age increased, age group (61-75 year) had BMD lower than that in

age group (45-60 year).

T-score increased as BMI increased, BMI < 25 had the lowest *T*-score while BMI > 30 had the highest *T*-score.

T-score decreased when the age increased, age group (61-75 year) had *T*-score lower than that in age group (45-60 year).

This study revealed that the severity of osteoporosis increases with age, as the BMD and *T*-score (which reflect the severity of osteoporosis) decline when the osteoporotic patients are getting older.

This result agrees with that of Fatayerji, et al. who pointed that there is a substantial decrease in BMD of the pelvis and proximal femur, sites rich in trabecular bone. These are the same sites associated with substantial increases in fracture incidence with aging [27].

Another study showed the same result, in which Tarek Fawzy, et al. concluded that advancing age is an important risk factor in the occurrence of low BMD [28].

In aging, the osteoclast activity is greater than osteoblast activity and results in net bone loss. The amount of bone formed during remodeling decrease with age in both sexes. The formation of osteoblast decreases and the rate of bone formation deceases, too, so bone mineral density decreases with increase formation of adipocytes in the bone marrow [29].

Although age-specific variations in bone density are mainly determined by genetic factors, but it also may be due to estrogen deficiency and vitamin D deficiency that is related to age [30].

There are other factors that contribute to bone osteoporosis with relation to increase age: Lack of exercise, prolonged immobilization from injuries, medications like corticosteroid and thyroxin, underlying disease states like metabolic dysfunction as hyperthyroidism, neuropathies, arthritis and gastrectomy. In addition to increase the risk factors of osteoporosis as smoking and alcohol consumption [29].

The serum leptin concentration increases with age. As the serum leptin concentration in age group (45-60 year) is lesser than that in age group (61-75 year).

Mann, et al. got the same result in their study. They found that in both sexes, the rise in leptin with age was associated with a decline in OB-R (leptin receptor), and age-related changes in both parameters preceded the pubertal rise in gonadal hormones. With age, serum leptin was higher and OB-R was lower in women. There was a significant negative correlation between OB-R and leptin in women [31].

Although the increase in plasma leptin concentration in aging may be partially attributed to the development of obesity (which is associated with leptin resistance), the increase in plasma leptin level during aging is often disproportionate to the increase in the amount of fat. It is therefore hypothesized that aging by itself is associated with a failure in leptin's action, independent of obesity or changes in body fat distribution. Thus, leptin resistance of aging may represent a perpetuating factor in developing and maintaining obesity and its clinical consequences. So serum leptin concentration increases with age mainly due to leptin resistance as OB-R (leptin receptors) declines with age [32].

Within the same BMI group (BMI < 25), there is a significant correlation between: BMD and *T*-score, BMD and age and between *T*-score and age. Leptin does not have any significant correlation with other parameters.

Regarding the correlation between the studied parameters within the same BMI group (BMI 25-30), there is a significant correlation between: BMD and *T*-score, BMD and age and between *T*-score and age. Leptin does not have any significant correlation with other parameters.

While in BMI group (25-30), the correlations between the studied parameters is significant correlation between: BMD and *T*-score, BMD and age, BMD and Leptin and between *T*-score and age. Leptin in this group has a significant correlation with age in

addition to BMD.

It is well established that the correlation between BMD and *T*-score is significant in all BMI groups, as both of them numerical value for osteoporosis severity [5, 6].

Age has a significant correlation with BMD and *T*-score in all BMI groups, as the study showed that both age and BMI has an impact on osteoporotic postmenopausal Iraqi women, but this significant correlation of age with BMD and *T*-score said that age has more impact than BMI on osteoporosis severity in this study.

Leptin has only a significant correlation with age and BMD in BMI group (BMI > 30). This could be due to the high serum Leptin concentration in this BMI group [23, 24].

The multiple comparison of studied parameters between the different BMI groups showed that there is a significant difference of BMD and *T*-score in group BMI (25-30) and group BMI > 30 form that in group BMI < 25, while there is no significant difference of BMD and *T*-score in the groups of BMI (25-30 and > 30). Leptin does not show any significant difference between the different BMI groups.

The correlation between the studied parameters (within the same group and between different groups) was significant between some parameters and was not significant between others.

The significant correlation of age with bone mineral density and *T*-score showed that age has a more impact than BMI on the severity of osteoporosis.

4. Conclusions

Both age and BMI have an impact on osteoporosis although age shows more impact on the severity of the disease than does BMI. Studying the direct impact of leptin on BMD may open the way in using new methods in treating and preventing the osteoporosis in patients with risk factors.

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The Antiviral Action of Polyhexamethylene Guanidine Derivatives

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Abstract: Although the PHMG (polyhexamethylene guanidine) and other oligomer guanidines are known as highly efficient biocides against a broad spectrum of microorganisms and eukaryotic cells, the cell protection by PHMG derivatives has been established firstly in this study. The antiviral protection was also exhibited after 15 min pretreatment of different cell cultures with low-concentration of PHMG salts. Monolayers of the continuous bovine tracheal cells culture (TCC) and primary culture of chicken embryo fibroblasts (FCE) were treated with aqueous solutions of PHMG chloride salts or PHMG succinate. The molecules of PHMG polycation adhered to the plasma membrane of the cells tested as they were treated with PHMG for 15-30 min. The viral material was added to the cell cultures after the wash-out carried out twice to rid of unbound PHMG. The viruses of *Equine herpesvirus type 1*, *Rhinotracheitis infectious bovine* and *Equine infectious anemia virus* were used. The protective effect from the cytopathic action of herpes and retroviruses was exhibited after 15 min pretreatment of cell monolayer with PHMG chloride at the TCC concentrations of 10^{-3} - 10^{-2}% and FCE concentrations of 10^{-5} - 10^{-4}%. The unique antiviral properties of PHMG salts represented in our research had never been shown before.

Key words: Polyhexamethylene guanidine, tracheal cell culture, fibroblasts, viruses.

1. Introduction

Polymeric guanidines are commonly used as antiseptics and disinfectants. The correct application of these biocides plays a profound role in the elimination of infection for in- and out-patient treatment and in veterinary [1-3]. The PHMG (polyhexamethylene guanidine), a member of the polymeric guanidine family, has broad-spectrum activity against Gram-positive and Gram-negative bacteria, fungi, yeasts and viruses.

PHMG is most effective against viruses which shell contains lipids, such as influenza, human immunodeficiency virus, Hepadnaviridae, Retroviridae, Caliciviridae, Orthomyxoviridae, Paramyxoviridae, Reoviridae, Picornaviridae, Adenoviridae, Parvoviridae groups and some others [4].

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PHMG hydrochloride is a highly water soluble polymer. It is odorless, colorless, noncorrosive [5] and much less toxic than common disinfectants [6] to humans and animals within a concentration range of 0.01%-1.0%. PHMG has been widely used for many years as antiseptic and disinfectant in Russia, Ukraine and other countries.

The net negative charge often stabilized by the presence of divalent cations such as Mg^{2+} and Ca^{2+}, it is the important characteristic of the outer envelope of different bacterial cells. Antimicrobial cationic polymers including PHMG interact with phospholipids of outer membrane and the plasma membrane (PM) of cell. PHMG can also interact with weakly charged membranes containing phosphatidylcholine that is typical for eukaryotic cells [7]. The PHMG adsorption is likely to change the surface charge of the cell membrane and the transmembrane potential, membrane permeability to water and small ions,

mobility of phospholipids and regular activity of enzymes.

It is possible to expect that changes of plasma membrane properties, caused by PHMG adsorption are capable of changing cellular sensitivity to viruses. The authors have expected that nontoxic for the cell concentrations of PHMG can protect cells from some viruses [7]. To demonstrate antiviral properties of PHMG, the authors used the primary culture of fibroblasts of the chicken embryo (FCE) and interwoven culture of the tracheal cells of calf (TCC).

2. Materials and Methods

2.1 Chemicals

The salts of PHMG chloride (•HCl) and PHMG succinate (•HOOC-C_2H_4-COOH) were kindly provided by "Termite" (Rivne, Ukraine). The PHMG was the mixture of its oligomers with *n* ranging from 2 to 20 and a mean value of 10. The salts of PHMG were dissolved in water, phosphate buffer (pH 7.2) and Hanks balanced salt solution at the volume ratio of 1:9. The water solutions of PHMG salts were used at the concentrations ranging from 1×10^{-7} to 1×10^{-2} % or 0.001 to 100 mg/L (or $\approx 10^{-9}$-10^{-4} mM).

2.2 Cell Cultures and Viruses

The primary culture of fibroblasts of the chicken embryo (FCE) and interweaved culture of the tracheal cells of calf (TCC); virus-liquids from group of Herpesviridae: *Equine herpesvirus type 1* (vaccine strain CB-69) and *Rhinotracheitis infectious bovine* (strain TK-A), from group of Retroviridae: *Equine infectious anemia virus* (field strain). The average activity of used virus strain was ≈ 100 lg TCD_{50}/cm^3.

2.3 Methods

The cell cultures and laboratory tests were made by standard methods with modifications [8, 9]. Cells were grown in the solution that contained a mixture of 199 medium (45%), a minimum Eagle medium or MEM (45%) and blood serum of cattle (10%). The monolayer was grown after seeding cell suspensions in 96-well plastic plates at 0.1 mL per well. PHMG solution at the concentrations of 0.1, 0.2 and 0.3 mL per well was placed into the plastic plates where the monolayer of cells was formed. The nutrient media was carefully removed from the monolayer before PHMG addition. PHMG was removed after 15 or 30 min preincubation with the cells. The viral material was added into the nutrient medium as monolayer cells were washed with sterile buffer solution and Hanks. The Hanks solution was kept with the cells for 15 or 30 min instead of PHMG in the control experiment No. 1. After pretreatment with PHMG and rinsing with the sterile buffer solution in control experiment No. 2 the Hanks solution was added to the monolayer of cells instead of the viral material. Plates with the monolayer of cells were incubated on air up to 5-7 days in the thermostat at 37 °C. The state of the cells monolayer at the determination of cytopathic viral action and cell destruction was estimated visually by the binocular laboratory microscope (magnification × 70).

3. Results and Discussion

The viruscides concentration of PHMG salt that neutralized the virus was determined upon the addition to viruses-containing suspension. This concentration was 10^{-5}% higher for PHMG chloride [7]. Short 15 min pretreatment of FCE monolayer formed with PHMG chloride solutions at the concentrations of 10^{-5}-10^{-4}% reliably protected the cells against viral infection of rhinopneumonia horses (*Equine herpesvirus type 1*). The results of these experiments are represented in Table 1.

PHMG showed a protective effect after washing the monolayer with a sterile buffer solution and Hanks. It is, therefore, possible to suggest that its molecules are closely connected with the PM of the cells and thus provide an antiviral protection. The virus in the environment surrounding of the cells remained intact. It is likely that in this case the PHMG-induced PM

Table 1 Antiviral effect of PHMG on fibroblasts of the chicken embryo (FCE).

The concentrations of PHMG chloride, %	Condition of the cell monolayer
10^{-4}	Monolayer remained unchanged during the 3 days of observation, the virus did not damage the cells
10^{-5}	Monolayer remained unchanged during the 3 days of observation, the virus did not damage the cells
10^{-6}	Monolayer began to damage within the first 24 h, there were areas of damaged cells, complete destruction of monolayer during the next 24 h, the virus damaged the cells
10^{-7}	During the first 7-12 h monolayer began to damage, there were some areas of destroyed cells, during 12-24 h subsequent destruction of the monolayer (~ 90%), on the next day the monolayer was fully destroyed, the virus damaged the cells
Control No. 1	Monolayer of cells affected by a virus was destroyed within 12-36 h of incubation
Control No. 2	Monolayer was normal, no changes found over 6 days time

$P > 0.95$.

Table 2 The action of virus on the tracheal cells of (TCC) calf after 15 min incubation with PHMG salines.

The concentrations of PHMG, %	Cell destruction by the virus, % damaged part of TCC monolayer					
	Equine infectious anemia virus		*Equine herpesvirus type 1*		*Rhinotracheitis infectious bovine*	
	in 2 days	in 6 days	in 2 days	in 6 days	in 2 days	in 6 days
PHMG succinate						
10^{-2}	0	0	10	50	0	40
10^{-3}	10	50	20	70	20	100
10^{-4}	30	100	30	100	40	100
10^{-5}	70	100	70	100	80	100
PHMG chloride						
10^{-2}	0	0	0	0	0	0
10^{-3}	0	0	0	0	0	0
10^{-4}	20	70	0	10	10	70
10^{-5}	30	100	30	100	20	100
Control	80	100	80	100	90	100

$P > 0.95$.

destruction never occurred. Cell monolayer was infected and destroyed during the first-second days of incubation as in the control No. 1 should this virus-containing solution was transferred to another one not treated with PHMG.

Chicken embryo fibroblasts are not quite adequate for the investigation of the action of herpes virus of horse rhinopneumonia and bovine rhinotracheitis. Pathogen rhinotracheitis has a pronounced tropism to epithelial cells of the mucous shells of the upper respiratory tract and genitals. Therefore, TCC cells were used for the next set of experiments. The results of the antiviral action of different PHMG salts concentrations on TCC monolayer after 15 min preincubation with PHMG salts are shown in Table 2.

PHMG chloride was found to protect the tracheal cells from cytopathic action of all tested viruses within 6 days at the concentrations of 10^{-3}-10^{-2}% after 15 min preincubation time. The concentration of 10^{-4}% showed only partial antiviral protection, while the lower concentrations (10^{-5}% or less) exhibited almost no cells protection against the viruses. The PHMG chloride antiviral action was insufficient on TCC at the concentrations of 10^{-5}-10^{-4}% that quite reliably protected fibroblasts against virus infection of horse rhinopneumonia. This could result from the structural differences of transformed tracheal cells PM, and their greater sensitivity towards the most adequate object, the virus of rhinopneumonia.

The virus protective action of PHMG succinate was lower. It showed full (*Equine infectious anemia virus*) or partial (*Equine herpesvirus type 1* and

Rhinotracheitis infectious bovine virus) protective effect only at the concentration of 10^{-2}%. At the lower concentrations PHMG succinate did not protect cells from damage. The antiviral protection was insignificant even though in some cases it was all else observed. Therefore, in the next set of experiments the time of TCC exposure to PHMG was increased from 15 min to 30 min. PHMG succinate was found to completely protect cells from damage by rhinopneumonia virus (Equine herpesvirus type 1) at a concentration of 10^{-2}%. The cytopathic effect of the virus was not shown within 6 days time interval. The other strains of viruses weren't tested in this study. PHMG succinate at the concentration of 10^{-3}% exhibited only ~50% of antiviral protection. Hence, the time of PHMG adsorption on the PM depends on the anionic composition of PHMG salts.

In conclusion, the obtained data suggest that antiviral protection by PHMG salts depends on their anionic composition (chloride, succinate or others), concentration and the incubation time. PHMG polycations bind membrane lipids and lipoproteins and block the adsorption of the virus. Thus, the antiviral action of PHMG is likely to have no species specificity since similar results have been obtained for different types of viruses.

Perhaps, the potential of the cell surface change due to increased ionic conductivity of the damaged membrane and the positive charge of the PHMG molecule. The screening of PM lipid receptors by PHMG polycation molecules impairs or even prevents the viruses from penetration into the cells. One may also suggest that adaptation stress caused by the adsorption of xenobiotic PHMG on the PM surface increases the overall cell resistance.

The analysis of the degradation of cell monolayer indicates that PHMG concentrations insufficient for the cell protection are still capable of temporary antiviral action that prevents cells from the damage by viruses. A similar short-term or semi-protective effect was shown at the application of specific antibodies or viral inhibitors. Perhaps, there is a gradual chemical degradation of the polymer structure or the phospholipids that lost their properties and functional significance after incubation with PHMG were rejected by the cell and removed from the cell PM.

The mechanism of antiviral action of PHMG is likely to be similar with its antimutagenic action [10]. In this research the authors checked on the possibility to induce the reverse mutation by the application of N-methyl-N1-nitro-N-nitrosoguanidine to some microorganisms like *Salmonella typhimurium*. The efficacy of PHMG protection revealed for this and other relatively strong mutagens was high enough as it even prevented the formation of auxotrophic mutants of pretreated cells. The authors believe that PHMG-induced membrane action that prevents the interaction of the mutagen with the intracellular content including DNA may underlie another mechanism of PHMG antimutagenic action.

All viruses tested contained lipids in their membranes. However, those viruses did not contain fully formed virions, only. Instead, there were viral particles with and without lipoprotein membrane formed. It also matters that the charge of polyanion (and not otherwise) of viral nucleic acid (RNA or DNA) is negated by electrostatic binding with PHMG polycation.

4. Conclusions

The antiviral protection of PHMG salts first revealed in this research may result from the adsorption of PHMG polycation to the plasma membrane of target cell and its strong binding with membrane phospholipids that screen the cell from virus. To display this effect the concentrations of PHMG must be higher than those for non-transformed FCE while processing transformed cells TCC. Short 15 min pretreatment by PHMG chloride at the concentrations of 10^{-5}-10^{-4}% protected the monolayer of fibroblasts against horse rhino pneumonia. PHMG chloride provided an antiviral protection of the

tracheal cells monolayer at the concentrations of 10^{-3}-10^{-2}% after 15 min preincubation whilst less efficient PHMG succinate protected cells at the concentration of 10^{-2}% after 30 min preincubation.

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Molecular Discrimination of Wild Philippine Paddy Straw Mushroom (*Volvariella volvacea*)

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Abstract: Genetic diversity study of wild Philippine paddy straw mushroom (*Volvariella volvacea*) was conducted to establish a germplasm collection accessible to volvariella producers in the Philippines. Forty one wild strains were collected from different geographical areas in Northern and Central Luzon region. Strains were differentiated using random amplified polymorphic DNA (RAPD). A single 10-based primer was used to generate randomly amplified polymorphic DNA (RAPD) in *V. volvacea* and differences were noted in band size (bp) ranging from 1,800 bp to 550 bp. Principal component analysis (PCA) of the RAPD data revealed 4 groups from wild strains. One strain showed RAPD pattern with band appearance at 1,750, 950 and 750 bp; 3 strains at 1,800 and 750 bp; 8 strains at 1,500 and 550; and the most abundant group with 29 strains at 750 bp. With observed lack of heterogeneity among strains, it is recommended that more collections from the wild should be undertaken for more diverse germplasm collection. Moreover, it is suggested that RAPD can be used to delineate strains of *V. volvacea* with potential importance on genetic diversity conservation and breeding.

Key words: *Volvariella volvacea*, genetic diversity, random amplified polymorphic DNA (RAPD).

1. Introduction

Volvariella volvacea, is a leaf-litter decomposing edible mushroom, commonly found growing saprophytically on decomposing piles of rice straw, banana leaves, coffee hull and sugarcane bagasse during the onset of the rainy season [1]. Growers in the villages of the Philippines usually cultivate this mushroom on bundled rehydrated rice straw or stubbles or banana leaves [2].

Three major problems usually encountered by paddy straw mushroom growers in the Philippines are the lack of practical technological expertise, availability and accessibility to spawn, and continuous availability of cultures. The first two problems have been properly addressed through our research efforts and regular training and extension programme being undertaken at the Centre for Tropical Mushroom Research and Development in the Philippines. However, sustainability and availability of viable cultures and its accessibility to farmers remains a major problem in sustaining *V. volvacea* production in the countryside. A need to continuously search and screen strains from the wild in order to ensure the availability of promising strains to growers is of paramount importance, since its still from the wild which is the best source of viable strains. Determining the genetic diversity among strains is of significance in order to delineate variation and serve as a basis for strain improvement [3]. Accessibility of promising strains to growers would ultimately lead to a more sustained year round production.

The conventional method of identifying and characterising strains of *V. volvacea* is based on the morphological and cultural characteristics of the

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isolates. These characteristics include the size, colour, weight, shape of the pileus and stipes, optimum temperature, pH and relative humidity for mycelial growth and fruiting. Although edible mushrooms like *V. volvacea* are conventionally characterised by the specific morphological characteristics of the fruiting bodies [4], it is impractical to always fruit different strains in order to characterise and identify them. This procedure is quite tedious and difficult when knowledge on the fruiting initiation mechanism is inadequate. To ease this problem, the use of available molecular methods to delineate the strains is recommended. With the use of this method, different strains can be characterised at a molecular level in a highly reproducible manner. Since its discovery, it has been used intensively in a wide range of applications [5].

Biodiversity is a key resource for cultivated edible mushroom improvement. To assess the biodiversity within species and to obtain useful genetic information for future breeding, it is necessary to use neutral markers as well as genetic markers [6]. Application of molecular genetic methods has allowed studies of the pattern of genetic variation in natural populations of fungi [7]. These recently applied methods allow polymorphisms of the nucleic acid and protein molecules providing additional insight into systematic relationships often useful for initial selection of lines [8]. Basic tools used to study genetic variations within and between populations are called genetic markers. Markers allow one to determine what alleles are present in populations, and are therefore very useful for studying a wide variety of questions in ecology and evolution [9]. These methods provide additional insight into systematic relationships often useful for initial selection of lines [8].

In this study, random amplified polymorphic DNA (RAPD) was used to differentiate strains of *V. volvacea*. In RAPD, randomly generated primers, 10 bases in length, are used for PCR with the entire genome of the study organism serving as a template. If a sequence complementary to the primer(s) occurs on both strands of DNA, then this fragment will be amplified and appear as band on the gel when the products of the PCR reaction are electrophoretically separated [7]. This PCR-based method avoids the need for restriction enzymes, blotting, probing or cloning and produces fragment differences similar to RFLP analysis. This analysis has been particularly popular for studies of population genetics and mapping of fungi [7].

2. Materials and Methods

2.1 Sources of Wild V. volvacea

Wild strains of *V. volvacea* were collected from different geographical areas in Central and Northern Luzon, Philippines. Collection was conducted during the rainy season when they are expected to produce fruit bodies. Samples were collected from different geographical locations in central and northern Luzon, Philippines (Fig. 1). Immediately after collection, sterile tissue of the fruit body was aseptically inoculated in a flat bottle with PDA and was incubated until its mycelia had fully ramified the culture medium. Cultures were revived and used as sources for molecular characterisation.

2.2 Revival of Cultures

Once the strains were collected, secondary mycelium were inoculated aseptically on to PDA in flat bottles and were incubated for seven days at room temperature (28-30 °C). This was to determine if the strains were viable. Revived cultures were labelled according to its source and stored.

2.3 Sample Preparation and DNA Extraction

Forty one wild strains were used in this study. Cultures were grown in 50 mL potato dextrose broth (FOrMedium LTD., Norwich, UK) in 250 mL conical flasks shaken at 200 rpm at 35 °C for 14 days. Flasks were inoculated using a 10 mm mycelial disc from a 7 day old PDA plated culture. Mycelium was harvested onto sterile muslin, washed with sterile water, excess

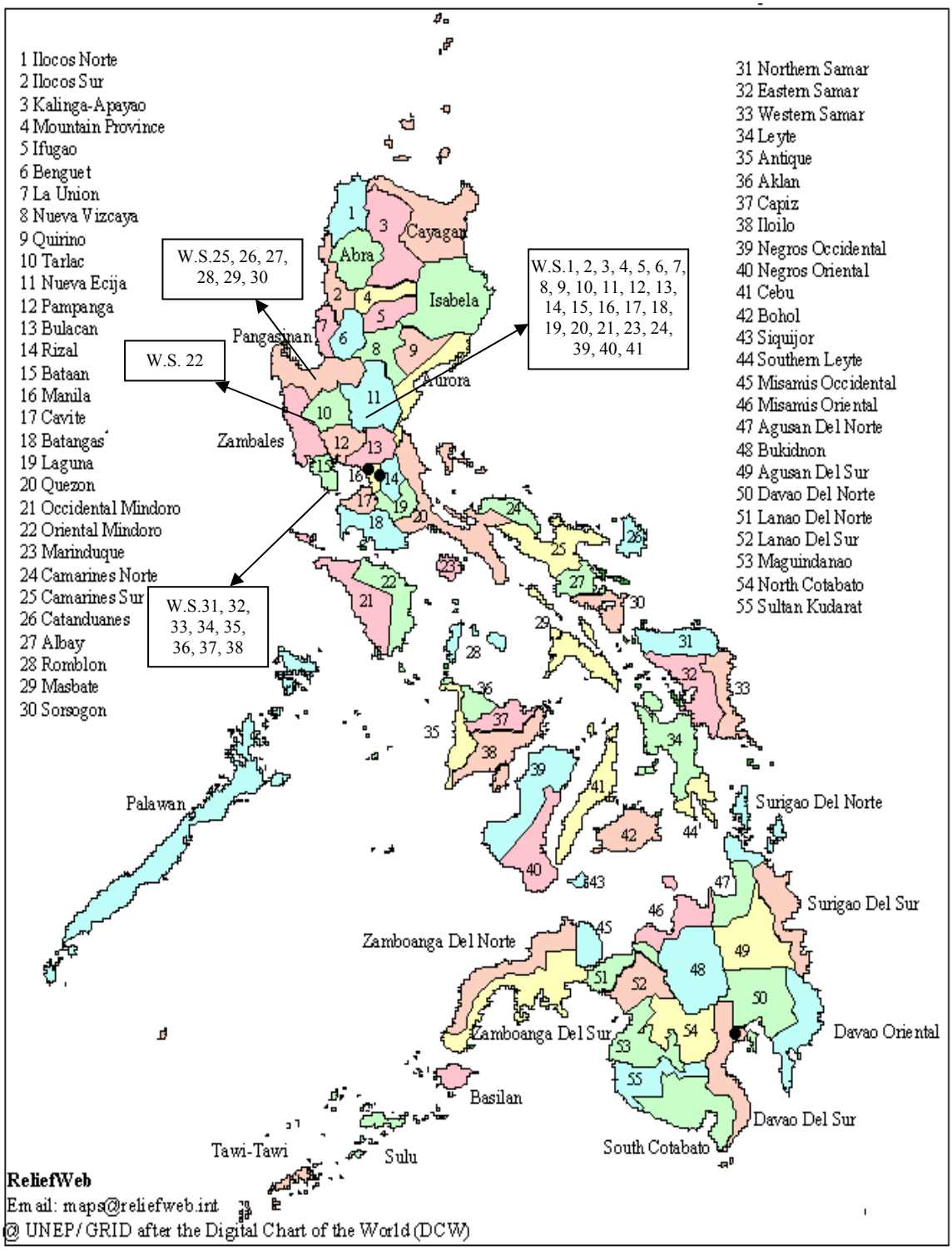

Fig. 1 Location map of origins of the collected commercial and wild *V. volvacea* strains.
Locations of origins of wild *V. volvariella* strains were determined using the global positioning system (GPS).

liquid removed by blotting and ground in a mortar and pestle in liquid nitrogen until a fine powder was obtained. DNA was extracted using a DNeasy Plant Mini Kit (Qiagen Ltd., Crawley, United Kingdom) according to the manufacturer's instructions. DNA was stored at -20 °C until required.

2.4 RAPD Method

Ten commercial random oligonucleotide primers (Invitrogen, UK) were screened for optimum number of RAPD fragments with sample strains (Primer S12 [CCT TGA CGC A], Primer6 [GAA ACA GCG G], Primer8 [GGA GCC CAC], Primer14 [GCC GTC TAC G], Primer17 [GGC ATC GGC C], Primer21 [GTG AGC GTC], PrimerOPL1 [GGC ATG ACC T], PrimerOPS5 [TTT GGG T], PrimerS1 [GTT TCG CTC C] and PrimerS10 [CTG CTG GGA C]). PrimerOPL1 was chosen for RAPD analysis on the basis that it produced the most number of DNA fragments visualised on gel electrophoresis. Each RAPD reaction was carried out in a final volume of 50 μL volume containing 0.50 μM primer OPL1 (GGC ATG ACC T), 2 mM $MgCl_2$, 200 μM of each of the 4 dNTPs, and 1.25 units of *Taq* DNA polymerase (Roche Diagnostics Ltd., Lewes, United Kingdom) and at least 10 ng of template DNA. Amplification was performed with initial denaturation at 92 °C for 5 min followed by 40 cycles at 94 °C for 1 min, annealing at 37 °C for 1 min, and 72 °C for 1.5 min, and final extension at 72 °C for 10 min. Amplified products were electrophoresed at 80 V through a 1.5% (w/v) flat bed agarose gel (Appligene, UK). Marker fragments of known molecular weight (1 kb ladder, Gibco BRL) were used to determine PCR product sizes by comparison with the marker. The running buffer used was 1 × TPE from a 10 × TPE stock [108 g Trizma base, 15 mL Orthophosphoric acid (BDH, Analar) and 40 mL 0.5 M EDTA in a total volume of 1 L]. A fifth volume of DNA loading buffer [0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 15% (v/v) Ficoll] was added to the PCR product prior to loading into the wells. The gel was stained with Ethidium bromide (20 μL of a 10 $mg \cdot mL^{-1}$ solution diluted in 500 mL 1× TPE buffer) for 30 min to visualize the DNA using a UV transilluminator. Gel photographs were taken using an UVl Tec gel imaging system.

2.5 Analysis

Principal component analysis (PCA) was determined using the Paleontological Statistics Software Package for Education and Data analysis [10].

3. Results and Discussion

V. volvacea strains were subjected to molecular characterisation using the Random amplified polymorphic DNA (RAPD) method. Differences were determined by the presence and absence of bands of specific base pairs (bp). Hyperladder I was used as the molecular marker for comparison. It was noted that bands were observed approximately from 1,800 to 550 bp. The DNA profile of wild strains revealed by RAPD is presented in Figs. 2-4. Strains 1-12; 17-27; 28-30 and 39-41 were found to be closely related with one band appearing after RAPD at 750 bp. Strains 13-15 showed bands at 1,800 and 750 bp, whereas strain 16 was unique with bands observed at 1,750, 950 and 750 bp. Strains 31-38 on the other hand, yielded bands at 1,500 and 550 bp. Principal component analysis (PCA) (Fig. 5) resolves four strain groups. Moreover, relating the collection sites (Fig. 1) with the RAPD patterns yielded (Figs. 2-4), strains coming from the same geographic location tend to be more closely related.

There are a number of reasons or factors that may cause differences in morphological characteristics of strains. It is likely that differences are the result of interaction between the environment, the nutrients available in the media or growing substrate, and the inherent characteristics of the strain and capability to utilise these nutrients. Determining whether the differences are environmentally or genetically influenced is difficult when using the conventional way of characterising mushrooms. It is in this context that molecular method was applied to delineate the wild strains of *V. volvacea* at molecular level.

Molecular evaluation of the strains suggests that there are four strains groups from forty one collected

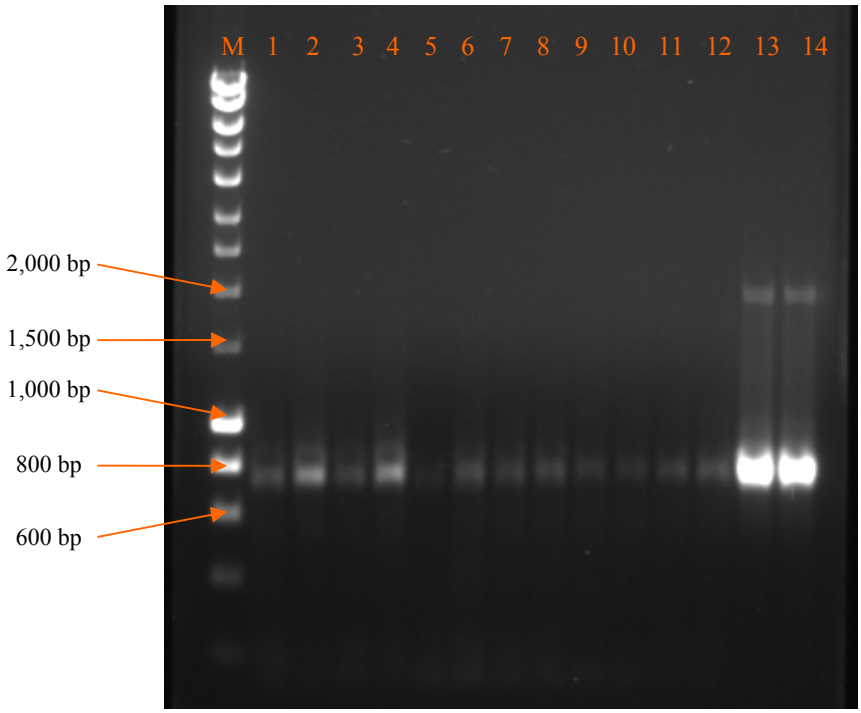

Fig. 2 RAPD patterns of wild *V. volvacea* strains (1-14).

Hyperladder I was used as molecular marker (M) as basis for determining the presence and absence of bands at specific base pairs (bp).

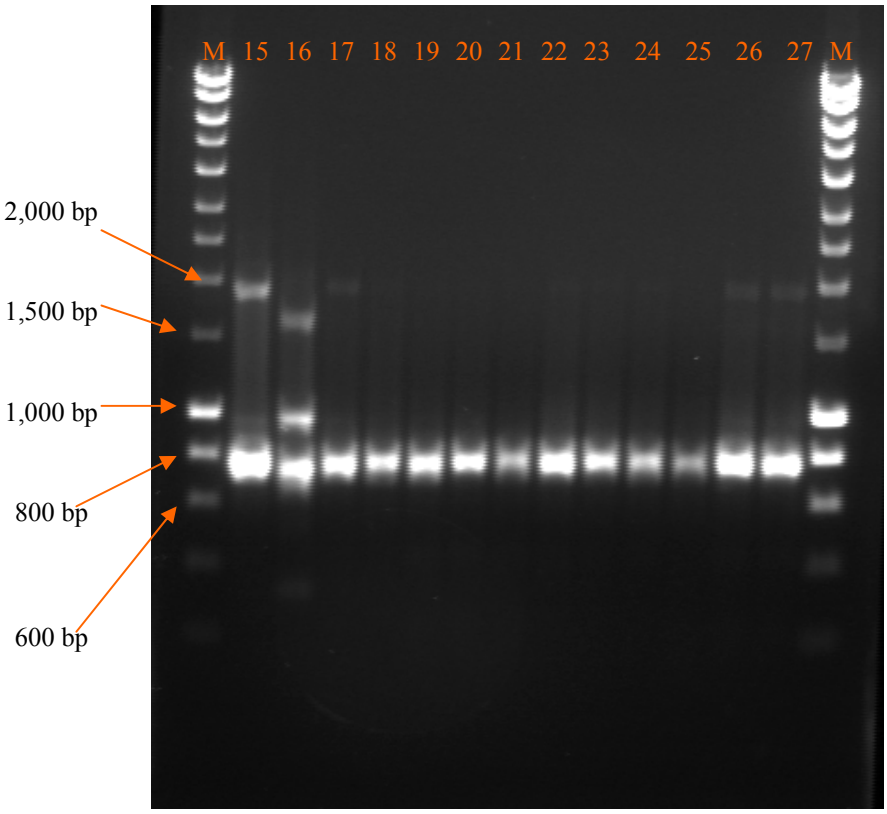

Fig. 3 RAPD patterns of wild *V. volvacea* strains (15-27).

Hyperladder was used as molecular marker (M) as basis for determining the presence and absence of bands at specific base pairs (bp).

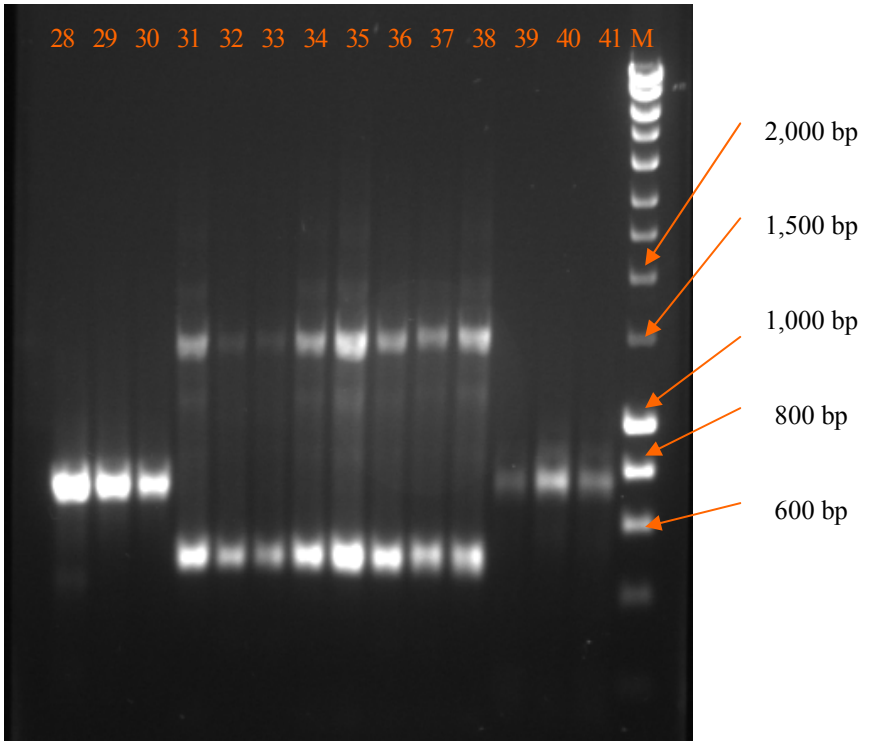

Fig. 4 RAPD patterns of wild *V. volvacea* strains (28-41).

Hyperladder I was used as molecular marker (M) as basis for determining the presence and absence of bands at specific base pairs (bp).

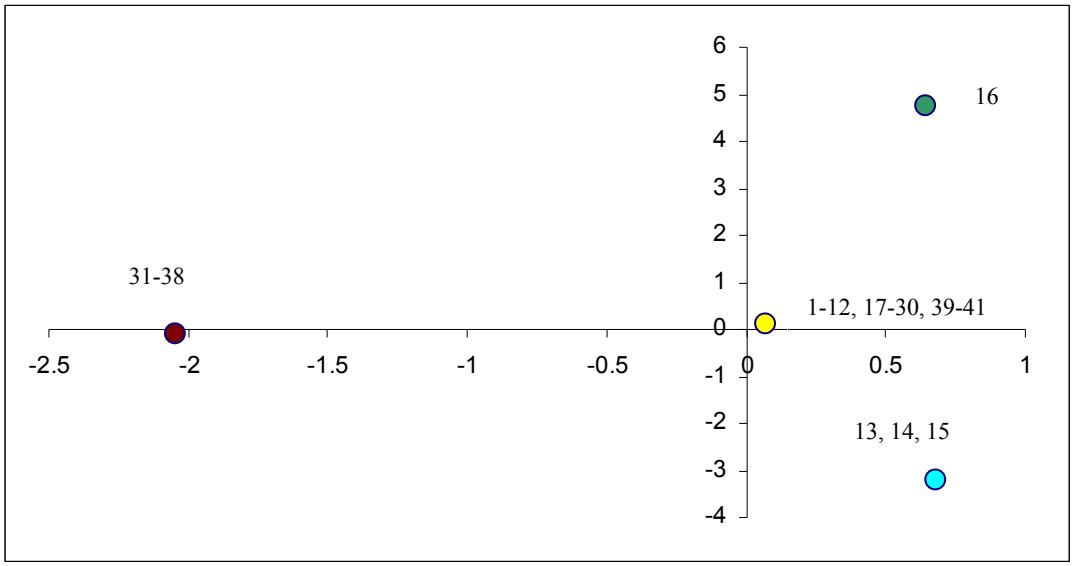

Fig. 5 Principal component analysis of wild *V. volvacea* strains' RAPD pattern. 4 strain groups were delineated as represented by the different colours.

wild strains. Looking at the spatial distribution of wild strains, they seemed to be more closely related when they were collected from within geographical area and tend to be different otherwise. There are few strains though, having same RAPD pattern across geographical locations. This parallels the findings of a study, when commercial strains of *Volvariella* from Malaysia, Philippines and Thailand was differentiated using RFLP. Results would suggest that no differences were noted among the commercial strains,

though they came from different geographical locations [11]. It was further cited that the observed highly similar DNA fingerprints reflect the lack of heterogeneity in commercial *V. volvacea* strains. In a study on the development of molecular and biochemical markers for selecting a potential high yielding strain of paddy straw mushroom (*V. volvavea*) reported that RAPD analysis of *V. volvacea* collected strains with common origin showed 85% to 95% similarity [12]. Collected strains of *Lentinula edodes* from different areas analayzed with RAPD revealed that strains showed similar RAPD patterns except for 2 strains [13]. High genetic homogeneity among cultivated strains of shiitake (*L. edodes*) in mainland China was also noted when they were analyzed using RAPD. It was recommended that collection from the wild should be encouraged since this mushroom is depending on a limited gene pool [14].

Volvariella volvacea like *Agaricus bisporus* is a self fertile species that does not require mating of two compatible species for fruiting to occur and is known as homothallism. Two common types of homothallism are found among self-fertile species, primary homothallism in which a homothallic mycelium is established from a single meiotic nucleus that has the potential to progress through dikaryons to completion of the sexual cycle, and secondary homothallism, in which a fertile dikaryotic mycelium is established from a basidiospore carrying two meiotic nuclei of different mating types. Secondary homothallism occurs in *Agaricus bisporus* while primary homothallism is common to *Volvariella* [15]. Unlike most basidiomycetes, mycelium produced by a uninucleate basidiospore of *V. volvacea* can complete the life-cycle producing a fruit body in which meiosis occurs. Since basidiospores are haploid and uninucleate, only one haploid set of chromosomes can occur in such a selfed (homothallic) fruit body and its progeny are expected to be identical to each other and to the parent [16]. Results on progeny analysis on genetical studies on the sexuality pattern of *V. volvacea* reveal that 90% of the monosporous isolates of mutants retained their marker's phenotype from parental phenotype. This proves that *V. volvacea* is a primary homothallic species [17]. This would probably explain why wild strains collected from within geographical areas tend to be more closely related.

4. Conclusions

With only four genetically different strain groups delineated from wild strains the diversity can be considered low. The lack of heterogeneity among wild population could be attributed to the homothallic nature of the organism. Since *V. volvacea* is considered to be primary homothallic, the chance of producing genetically different strains is low. It is essential that more collection efforts from the wild should be carried out to conserve variants within population and to have a more diverse germplasm collection. *Volvariella volvacea* production industry in the Philippines will primarily depend on crop improvement and available modern technology for higher production and income. Technology would always be available to growers; however, crop improvement of *V. volvacea* depends on the genetic resources availability. Genetic resources can be available only through genetic conservation and this is possible through exploring the wild and continuously collecting wild strains. This will facilitate the availability of strains for commercial and research and subsequently reduce the genetic vulnerability.

The natural genetic diversity and the present available technologies can be used in increasing the productivity of small scale mushroom farms [15].

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Chemical Composition and Antibacterial Activity of *Artemisia herba-alba* and *Mentha pulegium* Essential Oils

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Abstract: The chemical composition of essential oils obtained from *Artemisia herba-alba* and *Mentha pulegium* were determined. The essential oils were analyzed by gas chromatography coupled with mass spectrometry (GC/MS). Their antibacterial activity was studied *in vitro* against three standard strains: *E. coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, *Pseudomonas aeruginosa* ATCC 27853, and five clinical strains: *Enterobacter cloacae*, *Staphylococcus aureus*, *Pseudomonas pyocyanique*, *Enterococcus faecium*, and *E. coli*. Nineteen constituents were identified in *A. herba-alba* essential oil representing 99.57% of the total composition. The major component was α-thujone (59.07%). The bacterial strains were inhibited at concentrations ranging from 1.25 µL/mL to 5 µL/mL and killed at concentrations ranging from 1.25 µL/mL to 10 µL/mL. *M. pulegium* resulted in the identification of eighteen constituents representing 99.48% of the total composition. The main component was pulegone (78.07%). The minimal inhibitory (MIC) and bactericidal (MBC) concentrations were ranging from 1.25 µL/mL to 2.5 µL/mL.

Key words: *Artemisia herba-alba*, *Mentha pulegium*, GC/MS (gas chromatography-mass spectrometry), antibacterial activity.

1. Introduction

Therapy of bacterial infection is mainly based on the use of antibiotics. The widely and sometimes the inappropriate use of these agents, led to the development of multidrug resistant strains, hence the importance of directing research towards new ways especially toward herbal medicine that have always been a source of inspiration for new drugs.

Essential oils are volatile, natural, complex compounds characterized by a strong odor and are formed by aromatic plants as secondary metabolites [1]. They are widely used in medicine as constituents of different medical products, in the food industry as flavouring additives and also in cosmetics as fragrances [2].

Indeed, several studies have confirmed the antigenotoxic [3, 4], antibacterial [5, 6] and antifungal effects [7] of some essential oils and their components.

Artemisia herba-alba is a medicinal and aromatic dwarf shrub, that commonly grown in Mediterranean basin [8]. In Morocco, this plant is found in the Oriental regions, the Eastern Rif, the Middle Atlas, the High Atlas and the Saharan Anti-Atlas [9]. It is used extensively in traditional medicine to treat helminthiasis, diabetes mellitus and other

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conditions such as jaundice [10]. Also, the antihyperglycaemic [11], antimicrobial [12], antioxidant, antispasmodic, anti-venom, nematicidal, anthelmintic, anti-leishmanial, neurological, pesticidal and inhibitor activities of this plant have previously been reported [13].

Mentha pulegium is one of the *Mentha* species known as pennyroyal, a native herb of Asia and near East [14]. In Morocco, this plant grows in wet areas [9]. It has been traditionally used as antiseptic for treatment of cold, sinusitis, cholera, food poisoning, bronchitis and tuberculosis [15].

The aim of this study was to determine the chemical composition of essential oils of *A. herba-alba* and *M. pulegium* grown in Morocco and to investigate antibacterial activities against some clinical strains that exhibit multidrug resistance to commonly used antibiotics.

2. Material and Methods

2.1 Essential Oils

Essential oils used in this study were provided by Santis Company. They are extracted by steam distillation from flowers, leaves and stems (Table 1).

2.2 Microorganisms

The tested strains included the following bacteria: three standard strains: *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, *Pseudomonas aeruginosa* ATCC 27853 (Microbiology Laboratory, Faculty of Pharmacy, University of Barcelona, Spain) and five clinical isolates *Enterobacter cloacae*, *Staphylococcus aureus*, *Pseudomonas pyocyanique*, *Enterococcus faecium* and *Escherichia coli* (Microbiology Laboratory, CHU Ibn Rochd, Casablanca, Morocco).

2.3 Gas Chromatography-Mass Spectrometry Analysis

The chromatographic analysis of essential oils was performed with a gas chromatograph (Trace GC Ultra) coupled to a mass spectrometer (Polaris Q ion trap MS), with a VB-5 capillary column (methylpolysiloxane with 5% phenyl; 30 m × 0.25 mm; film thickness 0.25 µm). Fragmentation was performed by electron impact at 70 eV. Helium (1.4 mL/min) was used as carrier gas. Split-type injector was heated to a temperature of 200 °C. The volume injected was 1 µL. The column was initially maintained at a temperature of 40 °C for 2 min, increased to 180 °C at a rate of 4 °C/min, and finally raised to 300 °C for 2 min at 20 °C/min.

2.4 Disc Diffusion Method

The tested as described previously [16]. Sterile filter paper disc (6 mm diameter) were impregnated with 10 µL of essential oil and transferred into the Luria Bertoni Agar present in Petri dishes, which had previously seeded by spreading 1 mL of bacterial suspension adjusted to 10^6 CFU/mL. Standard antibiotics amoxicillin (25 µg/disk) were used as positive control. After incubation at 37 °C during 24 h, the diameters of inhibition zones were measured in millimeters. Tests were carried out in triplicate.

2.5 Determination of MIC and MBC

MIC was determined in this work by the method of macro-broth dilution [17]. A serial of dilution of essential oil ranging from 20 µL/mL to 0.15 µL/mL were prepared in test tubes containing Broth Luria Bertoni medium with 0.15% Agar [18]. Each tube was inoculated with the same volume of bacterial suspension adjusted to 10^6 CFU/mL. The tubes were then incubated at 37 °C for 18 h. MIC values were defined as the lowest concentration of the EO at which the absence of growth was recorded. Controls of medium with either microorganisms or the essential oil alone were included. From tubes where it was no trouble, aliquots of 10 µL were inoculated on Muller Hinton Agar medium. The MBC was the lowest concentration that gives no subculture. Each assay was repeated thrice.

3. Results and Discussion

3.1 Chemical Composition of Essential Oils

Analysis of the chemical composition of *A. herba-alba* essential oil by gas chromatography coupled with mass spectrometry (GC/MS) revealed the presence of nineteen compounds representing 99.57% of the total composition (Table 2).

Table 1 Region and period of collection of each plant studied.

Plant species	Region of collection	Period of collection
Artemesia herba-alba	Taroudant: Southeast Morocco	April-June 2009
Mentha pulegium	Taounate: Northeast Morocco	April-July 2009

Table 2 Chemical composition (%) of two essential oils from *Aremisia herba-alba* and *Mentha pulegium*.

Compunds	Percentage of compounds (%)	
	Artemisia herba-alba	*Mentha pulegium*
α-Pinene	0.68	0.41
Artemisia triene	3.75	
Sabinene	3.05	
2-Cyclohexen-1-ol, 1-methyl-4-(1-methylethyl)-, cis-	0.93	
ç-Terpinene	0.27	
α-Thujone	59.07	
2,4-Hexadiene, 2,3-dimethyl-	11.73	
α-CAMPHOLENE ALDEHYDE	12.71	
Bicyclo[3.1.1]hept-2-ene-2-ethanol, 6,6-dimethyl- (CAS)	0.72	
2-α-PINENE	0.25	
Patchoulane	0.30	
trans-2-Caren-4-ol	0.13	
α-Muurolene	0.16	
GERMACRENE-D	0.44	0.19
1H-Cycloprop[e]azulene, decahydro-1,1,7-trimethyl-4-methylene-, [1aR-(1aà,4aá,7à,7aá,7bà)]-	0.14	
1,1,3,3,5,5,7,7,9,9,11,11-DODECAMETHYL-HEXASILOXANE	5.24	13.37
3-Carene		0.29
(+)-Camphene		0.80
Cyclohexene,4-ethyl-3-ethylidene-4,8-		0.50
Bis(2-propylamino)-2,6-dichloro-1,5- naphoquinone		0.14
3-Cyclopentene-1-ethanol, 2,2,4-trimethyl-		0.35
l-MENTHONE		0.74
Isomenthone		0.36
Cyclohexanone, 5-methyl-2-(1-methylethenyl)-, trans		1.45
Pulegone		78.07
α-Cedrol		0.49
Bicyclo[5.2.0]nonane, 2-methylene-4,8,8-trimethyl-4-vinyl-		0.80
α-Caryophyllene		1.19
10aH-2,12a-Methano-1H,4H-cyclopropa[5,6][1,3]dioxolo[2',3']cyclopenta[1',2':9,10]cyclodeca[1,2-d][1,3]dioxin-15-ol, 1a,2,7a,13,14,14a-hexahydro-1,1,6,6,9,9,11,13-octamethyl-,acetate,[1aR-aà,2à,7aà,7bR*,10aà,12aà,13à,14aà,15S*)]-		0.13
10aH-2,12a-Methano-1H,4H-cyclopropa[5,6][1,3]dioxolo[2',3']cyclopenta[1',2':9,10]cyclodeca[1,2-d][1,3]dioxin-15-ol, 1a,2,7a,13,14,14a-hexahydro-1,1,6,6,9,9,11,13-octamethyl-, [1aR-(1aà,2à,7aà,7bS*,10aà,12aà,13à,14aà,15R*)]-		0.20
Total	99.57%	99.48%

Among these compounds, five of them can be considered as the main constituents: α-thujone (59.07%); campholene aldehyde (12.71%); 2,4-Hexadiene, 2,3-dimethyl- (11.73%); Artemisia triene (3.75%) and Sabinene (3.05%). The α-thujone was also identified as the majority constituents of the essential oil of Matmata in Tunisia (44%) [19] and Jordan (16%) [20].

The chemical composition of *Artemisia herba-alba* essential oil of Taroudant is vastly different from that of M'sila (Algeria), wich is dominated by camphor (19.4%) trans-pinocarveol (16.9%) chrysanthenone (15.8) and β-thujone (15%) [21]. Previous studies have shown that camphor was the main component of the *Artemisia herba-alba* of Algeria, Spain and Israel with a percentage between 15% and 68% [22-24].

The constituents of *Mentha pulegium* essential oil from Taounat are listed in Table 2. Chromatographic analyzes have identified eighteen compounds representing 99.48% of the total composition. The essential oil of *M. pulegium* is characterized by the presence of the pulegone as the main component with a percentage of 78.07%.

These results are similar to most of the work already done in Morocco [22-24]. Also, the work undertaken by Snoussi, et al. [25] and Hajlaoui, et al. [26] in Tunisia showed that pulegone was the major compound of *M. pulegium* with concentrations 44.27% and 61.11%, respectively. While work of Mahboubi, et al. [27] in Iran as well as those of Derwich, et al. [28] in Morocco highlighted another chemotype whose major compounds are piperitone and piperitenone with low levels of pulegone. In addition to pulegone (43.3% to 87.3%), Beghidji, et al. [29] found in different sources of Algeria, a chemotype of *M. pulegium* characterized by its richness in monoterpene (α and β-pinene, camphene, sabinene, α-terpinene and myrcene).

The chemical composition of *Artemisia herba-alba* and *Mentha pulegium* essential oils shows a large interspecies variability, due to climatic and soil variations, to the vegetative cycle, and to seasonal variation.

3.2 Antibacterial Activity

The *in vitro* antimicrobial activity of *A. herba-alba* and *M. pulegium* essential oils against microorganisms was qualitatively and quantitatively assessed by the diameter of inhibition zone, MIC and MBC values.

To determine the MIC and MBC, the authors adopted the method of broth dilution using 0.15% agar to ensure the homogeneity of the oil-water mixture [33]. The diameter of inhibition zone, MIC and MBC results are shown in Tables 3 and 4.

The data indicated that the oils exhibited varying levels of antimicrobial activity against the investigated bacteria. With the exception of *Pseudomonas* strains that shows resistance to the bactericidal and bacteriostatic action of *A. herba-alba* and *M. pulegium* essential oils, all other bacteria are inhibited at concentrations ranging from 1.25 μL/mL to 5 μL/mL for essential oil of *A. herba-alba*, and from 1.25

Table 3 Diameter of inhibition zone in (mm).

	Microorganism species	Inhibition zone diameter in (mm)		Positive control (AMX)
		A. herba-alba	*M. pulegium*	
Gram−	*E. coli* ATCC 25922	16 ± 0	15 ± 0	25 ± 0
	E. coli clinique	17 ± 0	12.5 ± 0.7	0 ± 0
	Ps. aeruginosa ATCC 27853	0 ± 0	0 ± 0	0 ± 0
	Ps. pyocyanique	8 ± 0	0 ± 0	0 ± 0
	Enterobacter cloacae	13 ± 0	11 ± 0	12 ± 0
Gram+	*St. aureus clinique*	11 ± 0	12 ± 0	12 ± 0
	St. aureus ATCC 29213	13 ± 0	15 ± 0	16 ± 0
	Enterococcus faecium	12 ± 0	12 ± 0	18 ± 0

Table 4 MIC and MBC of essential oils.

	Microorganism species	MIC (μL/mL)		MBC (μL/mL)		MBC/MIC	
		A. herba-alba	*M. pulegium*	*A. herba-alba*	*M. pulegium*	*A. herba-alba*	*M. pulegium*
Gram−	*E. coli* ATCC 25922	1.25	1.25	1.25	1.25	1	1
	E. coli clinique	2.5	1.25	2.5	1.25	1	1
	Ps. aeruginosa ATCC 27853	>20	>20	>20	>20		
	Ps. pyocyanique	>20	>20	>20	>20		
	Enterobacter cloacae	1.25	2.5	1.25	2.5	1	1
Gram+	*St. aureus clinique*	2.5	1.25	5	1.25	2	1
	St. aureus ATCC 29213	2.5	1.25	5	1.25	2	1
	Enterococcus faecium	5	2.5	10	2.5	2	1

μL/mL to 2.5 μL/mL for the essential oil of *M. pulegium*. And killed at concentrations ranging from 1.25 μL/mL to 10 μL/mL for essential oil of *A. herba-alba* and from 1.25 μL/mL to 2.5 μL/mL for the essential oil of *M. pulegium*.

Ps. aeruginosa which proved resistant to the antibacterial effect of the essential oils tested is known by a high level of intrinsic resistance to virtually all known antimicrobial compounds including essential oils [34, 35]. This resistance seems to be related with the nature of the outer membrane which is composed of lipopolysaccharides that form an impermeable barrier to hydrophobic compounds [36, 37], but this was not true about *A. herba-alba* essential oil which showed a strong antibacterial effect on Gram− bacteria.

Among the gram-positive bacteria, *Enterococcus faecium* was less sensitive to the action of the essential oils tested with MIC = 5 μL/mL for *A. herba-alba* essential oil and MIC = 2.5 μL/mL for *M. pulegium* essential oil, while *E. coli* ATCC 25922 was more sensitive with MIC = 1.25 μL/mL for both essential oils tested.

The MBC/MIC ratio is used to classify antibiotics according to their characters as bactericides (close to 1) or bacteriostatic (greater than 4) [38]. Our results showed that *A. herba-alba* and *M. pulegium* essential oils has a bactericidal activity against all bacteria tested excepted *Pseudomonas*. The presence of pulegone in *M. pulegium* essential oil and α-thujone in *A. herba-alba* essential oil may be responsible for their antibacterial activity. Indeed, it has reported that pulegone play an important role in antibacterial activity [39, 40]. However, Other Artemisia oils rich in camphor and 1,8-cineole were previously demonstrated to have potent antimicrobial activities in vitro [41, 42]. Thus, it is difficult to attribute the activity of a complex mixture to a single or particular constituent. Major or trace compounds might give rise to the antimicrobial activity exhibited. Possible synergistic and antagonistic effect of compounds in the oil should also be taken into consideration.

4. Conclusions

In this work, we studied the chemical composition and antibacterial activity of the essential oil of *Artemisia herba-alba* from Taroudant and *Mentha pulegium* from Taounat. Chemical analysis by GC/MS identified respectively nineteen and eighteen constituents. The α-thujone (59.07%) was the major component of *A. herba-alba* while *M. pulegium* was dominated by pulegone (78.07%). The results obtained in this study show that the essential oils tested in vitro have significant activity against most bacteria tested.

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The Importance of Consuming Charales (*Chirostoma jordani*) for Human Nutrition

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Abstract: The diversity of existing food may decrease malnutrition through the consumption of underutilized species. In Mexico, the "charal" fish *Chirostoma* spp. is grouped in five species: *Chirostoma grandocule*, *Chirostoma patzcuaro*, *Chirostoma humboltianum*, *Chirostoma attenuatum* and *Chirostoma jordani* which live in lakes in the country, and they are not consumed or their demand is low. The objective of this research was to analyze the macronutrients of *Chirostoma jordani* charal and inform population their nutritional value to increase human nutrition. Sampling was provided at Xochimilco channels in Xochimilco, D.F., summer and winter seasons at 2009. Fish (200 g) were maintained in channels water for proximal analysis according AOAC methods (1995). The results in dry bases were: protein 74.36%, lipids 1.24%, fiber 0.27%, minerals 4.9%, and soluble carbohydrates 19.23%. This fish grants protein, plastic material essential for human development: its lipid and carbohydrate contents, sources of energy, are low; however, the excess of disseminated proteins increase the energy sources. Fiber is found in a minimum amount. Dehydrated charales may be stored without refrigeration up to 3 months, maintaining their nutritional value. Consumption of the charal should be considered in the basic diet for its nutritional properties, to diminish malnutrition in the Mexico and other countries.

Key words: Nutrition, Charales fish, *Chirostoma jordani*, macronutrients.

1. Introduction

According to the FAO [1], almost 30% of the population worldwide suffers some type of malnutrition, either those who do not have access to a sufficient amount of energy or basic nutrients, or those who suffer diseases due to excessive and/or unbalanced eating. The deficiency in the intake of food with nutritional value unchains a series of chronic diseases which are increasing around the world [2]. It has been calculated that in 2001 these diseases caused approximately 60% of the deaths notified in the world and 46% caused a global load of morbidity, being malnutrition considered within the main causes of mortality [3]. Nevertheless, malnutrition can modify some intermediate mechanisms, such as cardiac function, metabolizing lipids or glucose, causing several diseases and even death [4]. For this reason, it is highly important to find natural origin foods that provide macro-nutrients of high nutritional value that, when consumed, complement a balanced diet. In Mexico there are a large number of these foods, such as the "charal", fish *Chirostoma* spp. by its scientific name. It is grouped in five species: *Chirostoma grandocule*, *Chirostoma patzcuaro*, *Chirostoma humboltianum*, *Chirostoma attenuatum* and *Chirostoma jordani*, these fishes inhabit in lakes, mainly in the central part of the country. These little fish live in fresh, clear water, are silvery white, they are 5 cm to 12 cm long and an average weight of 12 g.

Since prehispanic times, charales have played an important role in the feeding of Mexicans; however, the consumption of this species has decreased in time, for official surveys reveal that the consumption

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per capita of this fish between 1992 and 2002 was 12.72 kg/hab [5]; however, these numbers are lower than the minimum requirements that are 20 kg per capita per year [6]. In Mexico, *Charales* spp. is found within the six species that support fisheries, according to Olmos [7]. Nevertheless, this resource lacks acceptance among the population, for its consumption is not homogeneous, despite being reported as a highly functional food, with biologic and nutritional quality, and its consumption brings multiple benefits to health, beyond conventional nutrients [8].

The objective of the present study was to determine the macro-nutrient and mineral content present in *Chirostoma jordani*, to propose to the general population the consumption of this fish as a daily food, with a good content of nutrients and as a source of natural energy.

2. Method

The sampling area was the canals of the lakes in the municipality of Xochimilco, Mexico City; the weather is tropical, with an average temperature of 16 °C, and two climatic seasons were identified: dry season from January to May, and rainy season from June to September [9]. The samples were captured during January and July 2009, using 30 m long water nets with an 8.0 mm mesh, 2 m deep. Six samples at four different canals each of 200 g were gathered and analyzed in duplicate. The obtained samples were transported in glass container with water from their environment, at a stable temperature of 4 °C until they were processed, to the Food Science Laboratory at the Universidad Autónoma Metropolitana, Campus Xochimilco. With this material, taxonomic identification of the species was performed and the proximal analysis according to the AOAC methods [10]. The samples were dried at 60 °C for 24 h and ground to a fine powder for further determination of protein, ash lipid and crude fiber. Proteins were calculated from nitrogen content by Kjeldhal method using the conversion factor 6.25. Ash was determined by incinerating at 650 °C in a furnace muffle for 3 h. Lipids were determined by an extraction process with petroleum ether (b.p. 60-80 °C) at 120 °C for 6 h using a Goldfish apparatus. Crude fiber by acid hydrolysis followed by alkaline hydrolysis; the total carbohydrates content were calculated by difference [100 – (protein + lipids + ash + crude fiber)].

3. Results

Charales are classified as vertebrate, class Actinopterygii, order Atheriniformes, family Atherinopsidae, genus *Chirostoma*, specie *Chirostoma jordani* (Table 1), the moisture content is under 50% (Table 2).

Protein levels are high, the mineral content represent a high concentration of metal ions, thus these must to be assessed to determine the presence of some specific essential ions, carbohydrates content is high as well (Table 3). Edible portion used only, no bones analyzed.

4. Discussion

According to the results obtained from the proximal analysis of *Chirostoma jordani*, it was found that the amount of protein, an essential nutrient in human nutrition, was present in a high proportion, followed by soluble carbohydrates. Lipid and fiber contents were lesser and the percentage of minerals present in the sample was significant. In this way, charales are characterized mainly for being an important source of proteins of high biological value, for when consuming around 200 g of charales, they grant the following amino acids: valine, threonine, leucine, isoleucine, lysine and tryptophan [11] and the importance of their consumption is that they constitute the plastic material indispensable for human development, in addition of forming part of a low-fat diet, rich in protein [12]. This is paramount, for it has been demonstrated that the implementation of these types of diets constitute one of the main forms to fight malnutrition, currently

Table 1 Taxonomic determination of the charal (*Chirostoma jordani* Woolman, 1894).

Order	Atheriniformes
Family	Atherinopsidae
Genus	*Chirostoma*
Species	*Jordani*
Common name	Charal

Table 2 Moisture content in Charales, *Chirostoma jordani*.

Water: 41.12 %	Dry sample: 58.88%

Table 3 Proximal analysis of macronutrients in Charales, *Chirostoma jordani*, g/100 g dry basis.

Proteins*	Lipids	Minerals	Crude fiber	Soluble carbohydrates
74.369	1.24	4.90	0.27	19.23

*Protein = Kjeldhal N × 6.25. $n = 3$, mean reported $P < 0.05$.

considered a leading worldwide problem, which causes mortality and morbidity in different countries and in all sectors of the populations [13]. Data reported by Blouet et al. [12] and Belobrajdic et al. [14] establish that the diets given to patients with malnutrition problems act directly in the body by increasing the energy expenditure and the feeling of satiety, which contributes in a short term to improve human nutrition.

Even more, several studies indicate that the metabolism of proteins and consequently the energy consumption depends on the source from which they originate, for some of the factors that determine the metabolism of these macromolecules is their absorption rate and their amino acid composition, which is determined by the wide variety of carbon chains and cofactors which are derived from the catabolism of these [15]. Another mechanism due to the increase of protein intake in the diet is the suppression of hyperinsulinemia or insulin secretion, thus blocking the storage of carbohydrates in the body, leading to a balanced diet. Hence, implementing charales to diets designed to combat malnutrition problems is a viable alternative due to the high protein content versus low proportions of fat present in this *Chirostoma jordani*.

It is important to point out that recommencing the intake of *Chirostoma jordani* in the daily diet, represents enormous benefits to health, for according to Stamler [3] and Latham [16], the increase in chronic diseases worldwide are largely due to unbalanced food consumption. The lipid content in *Chirosmoma jordani* is found in low proportions and these fish are rich in omega-3 fatty acid important in health, as the intake of this oil from aquatic species contributes to lower blood triglycerides, and cholesterol, in addition to lowering vascular pressure and having antithrombotic and anti-inflammatory [17].

The low fiber content in *Chirostoma jordani* is another factor that contributes to consider this species as an excellent food choice for people with malnutrition, in addition to its composition of lean meat which makes it easy to digest. The mineral content in *Chirostoma jordani* was 4.9%, the consumption of these very important since their intake is essential for the nervous system development. The contribution of soluble carbohydrates, source of energy, is slightly higher than the recommended daily requirements; however this excess is not significant.

In some communities, it is customary to process charales, removing the viscera from the fish and covering it with small amounts of salt to be placed to direct sunlight for 3 days, obtaining a dry product that can be kept without refrigeration for up to 3 months retaining their nutritional value.

The excellent nutritional properties of this species are the main reasons for implementing its daily intake

of the general population, for its frequent intake may contribute to reduce malnutrition in the Mexican population and in other nations. In addition, it represents a consumer alternative that would reduce over exploitation directed only to certain types of seafood such as shrimp, tilapia, being these species the most in demand [5].

5. Conclusions

To implement the consumption of charales among the population represents an important source mainly of proteins, and in the diet of persons with malnutrition they constitute an essential food for a balanced diet. In addition, de acquisition of this highly functional food is feasible among different population sectors for it is available throughout the year and thanks to its preservation flexibility.

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Effect of Weed Control on Establishment and Herbage Production in Dwarf Napiergrass

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Abstract: Weed control is a crucial factor for maintaining establishment and herbage production in dwarf variety of late-heading type (DL) napiergrass (*Pennisetum purpureum* Schumach) in southern Kyushu, Japan. This study was aimed to verify the weeding-effect on dry matter (DM) production in the farm level and to examine the effect of several weed control, i.e. mixed sowing of annual setaria (abbreviated as S), which has no regrowth ability after stem-elongation, paper-mulching (as P) and hand-weeding (as +W), compared with no-weeding (as −W) on DM yield and quality of DL napiergrass in two years. Weed control practices significantly ($P < 0.05$) promoted several plant growth attributes in DL napiergrass, compared with no-weeding both in the farm and experimental levels. Paper-mulchidng (P-W or P+S-W) had highest yields among several practices in both years. Setaria-sowing ha a partially mitigating effect of weed damage on DM production of DL napiergrass, while additive DM gain from setaria could compensate the yield decrease in DL napiergrass and reduce herbicide cost. Neither digestibility nor crude protein was affected by any weed control in either year. Thus, paper-mulching and annual setaria-sowing could be effective alternative practices for weed control of this species.

Key words: Annual setaria, dwarf napiergrass, herbage yield, paper mulch, weed control.

1. Introduction

Weed invasion into forage fields is a visible sign of management problems. Damages to forage crop production by weeds are derived principally from loss in dry matter (DM) production and secondarily from deterioration of forage quality. However, use of herbicide should be avoidable due to care for its poison to animals and increase in herbage production cost [1].

Dwarf variety of late-heading type (DL) napiergrass (*Pennisetum purpureum* Schumach) was extended its cultivation area to several sites in southern Kyushu [2]. Through this extension activity of this species, weed control was crucial to obtain good establishment and considerable herbage production. Hand-weeding was a sole weeding practice for smallholder farmers, while it caused physical and spiritual burden for the farmers to maintain suitable establishment of this species. Therefore, easy and environmentally effective weed control practices should be developed.

Inter-row space of DL napiergrass is normally weeded by hand-mowing machine 2-3 times after transplanting before the pasture starts to be defoliated. Repeated weeding is essential until the leaf canopy of this species is well developed. Even though close spacing is more suitable for protecting weed growth, weeds do invade even at 0.5 × 0.5 m spacing of this species.

Mulching at the inter- and intra-row spaces reduces weed problems by preventing the seed germination and suppressing growth of emerged weed seedlings, resulting in facilitating soil fertility and plant productivity [3-5]. Mulching is a well-known method for the establishment of horticulture crop such as

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lettuce [6] and tomato [7] and also in paddy rice field [8]. In the temperate grass production, mulching is often used as living mulch or cover crop such as white clover [9], legume [10] and hairy vetch [11]. However, paper-mulching has not been applied to DL napiergrass as weed control management.

The other way for weed control is the oversowing of annual grass species to compete weed at the early growth of perennial forage crops. DL napiergrass was oversown with temperate Italian ryegrass (*Lolium multiflorum* Lam.) in the previous late-autumn to be harvested in the spring-early summer season [12]. Tropical annual setaria (*Setaria italica* cv. Natsukanso), released from Yukijirushi Seed Co. Ltd., is utilized as once-cutting herbage with no regrowth ability if it starts stem elongation at the harvest, gives early summer growth, and should be also ideal to suppress summer weeds at the early growth stage of perennial forage species [13].

Therefore, the objectives of this study were to verify the weeding-effect on DM production in the extension field and to examine the effect of weed control on establishment and herbage yield and quality in DL napiergrass by paper-mulching, oversowing of annual setaria and several weeding practices, compared with no-weeding control in two years.

2. Materials and Methods

2.1 Effect of Weeding on Establishment of DL Napiergrass in the Farm Level

The study was conducted at a beef-cow breeding farm in Miyazaki (31.93° N; 131.25° E) on brown forest soils from June to September 2013. DL napiergrass was established by transplanting rooted tillers at 1 plant/m^2 (1 × 1 m grid) in the two terrace soils sized at 100 m^2, each on 6 June 2013. Treatments consisted of the two weeding levels, in the weeding plots where weeding was conducted into the inter-row space twice on 3 July and 5 August 2013, and non-weeding plot where no-weeding was imposed after transplanting, and replicated five times in a completely randomized design for a total of 10 plots (20 m^2, each). Prior to defoliation the plant height, tiller number and DM of each plant fraction in DL napiergrass and plant height and DM of weeds were measured at 1 plant per plot and establishment of DL napiergrass plants were measured for whole plants on September 26, 2013.

2.2 Effect of Several Weed Control Practices on Establishment and Herbage Production

In 2008, DL napiergrass transplanted at 2 plants/m^2 (1 × 0.5 m spacing) was imposed on sandy Regosols by three treatments (P-W, S+W, S-W) replicated three times by randomized block design in Sumiyoshi Livestock Science Station (31.98° N; 131.46° E), University of Miyazaki (Trial-1). In 2010, DL napiergrass transplanted at 2 plants/m^2 (1 × 0.5 m spacing) was imposed on brown Ando soils by four treatments (P+S-W, S+W, S-W, -W) replicated three times by randomized block design in Kibana Agricultural Science Station (31.83° N; 131.41° E), University of Miyazaki (Trial-2). Growth attributes including DM yield, *in vitro* DM digestibility (IVDMD) and crude protein (CP) content as herbage quality were determined in both trials. Efficiency of weed control practices in plant parameters, such DM yield, IVDMD and CP content was evaluated by the percentage of plant parameter value in each weed control practice to that in non-weeding control.

3. Results and Discussion

3.1 Effect of Weeding on Establishment of DL Napiergrass in the Farm Level

Weeding stimulated plant growth of DL napiergrass in tiller density, DM yield and leaf blade percentage significantly ($P < 0.05$) and tended to increase establishment percentage, even though plants tended to elongate in non-weeding plots, probably due to competition of the napiergrass with weeds. Weed biomass was completely nurtured under non-weeding management, which reduced the tillering of

napiergrass after establishment and increased the risk of vulnerability (Table 1).

3.2 Effect of Several Weed Control Practices on Establishment and Herbage Production

Weed control management had significantly ($P < 0.05$) positive effects on tiller density and DM yield in DL napiergrass, compared with no-weeding (S-W or –W), and paper-mulching (P-W or P+S-W) had highest tiller density and DM yields in both trials, except for the 2nd defoliation in Trial 2 (Table 2).

Table 1 Effect of weeding on plant attributes in DL napiergrass and weed on 26 September 2013.

Plant species	Plant attribute	Treatment		Percentage of non-weeded to weeded	Significance†
		Weeded	Non-weeded		
DL napiergrass	Plant height (cm)	137.4	147.4	107.3	
	Tiller (No./m^2)	27.0	5.8	21.5	$P < 0.05$
	DM yield (g/m^2)	494.6	70.0	14.2	$P < 0.05$
	Leaf blade percentage	66.3	58.0	87.5	
Weed	Percentage of establishment	98.0	91.7	93.6	
	Plant height (cm)	137.3	158.7	115.6	
	DM yield (g/m^2)	201.8	497.6	246.6	

† By student's t-test.

Table 2 Effect of several weed control management on yield and quality attributes of DL napiergrass at the defoliations in Trial 1 (a) and Trial 2 (b).

(a) Trial 1 in 2008

Defoliation	Yield and quality attribute†	Treatment		
		P-W	S+W	S-W
1st	Plant height (cm)	81 a††	59 b	72 a
	Tiller density (No./m^2)	68 a	18 b	9 b
	DM yield (g/m^2)	121 a	17 b	7 b
	IVDMD (mg/g)	737	758	728
	CP content (mg/g)	215	226	233
2nd	Plant height (cm)	135 a	89 b	49 c
	Tiller density (No./m^2)	60 a	26 b	6 c
	DM yield (g/m^2)	121 a	17 b	7 b
	IVDMD (mg/g)	547	664	585
	CP content (mg/g)	86	95	112

(b) Trial 2 in 2010

Defoliation	Yield and quality attribute	Treatment			
		P+S-W	S+W	S-W	-W
1st	Plant height (cm)	122 a	105 b	119 a	118 a
	Tiller density (No./m^2)	122 a	57 b	19 b	18 b
	DM yield (g/m^2)	482 a	238 b	143 b	153 b
	IVDMD (mg/g)	660	653	616	616
	CP content (mg/g)	93	79	100	91
2nd	Plant height (cm)	129	125	112	114
	Tiller density (No./m^2)	120 a	96 a	68 b	70 b
	DM yield (g/m^2)	461	458	366	278
	IVDMD (mg/g)	619	622	628	630
	CP content (mg/g)	101	93	97	94

† DM yield, dry matter yield; IVDMD, *in vitro* dry matter digestibility; CP content, crude protein content. †† Figures with different letters denote significant difference by LSD ($P < 0.05$).

Table 3 Effect of weed control practices on efficiency in several parameters of plant total in DL napiergrass at each defoliation in 2008 and 2010.

Year	Defoliation	Parameter†	Practice		
			Weeding	Paper-mulching	Setaria-sowing
2008 (Trial 1)	1st	DM Yield	248	2,466	—
		IVDMD	4	1	—
		CP content	2	-9	—
	2nd	DM Yield	7,554	54,091	—
		IVDMD	14	-6	—
		CP content	-16	-24	—
2010 (Trial 2)	1st	DM Yield	74	275	-4
		IVDMD	6	7	1
		CP content	-19	-6	11
	2nd	DM Yield	73	73	36
		IVDMD	-1	-1	0
		CP content	-3	9	3

†DM yield, dry matter yield; IVDMD, *in vitro* dry matter digestibility; CP content, crude protein content.

Adoption of several weeding practices such as weeding, paper-mulching and setaria-sowing can be assessed by the percentage of gain or loss in attributes under the particular practice relative to those under no adoption of the practice (Table 3). Adoption of paper-mulching facilitated to obtain largest positive gain of DM yield in DL napiergrass at both defoliations in both Trials 1 and 2. Situation was similar for the positive gain by adoption of hand-weeding, while the degree of gain was reduced from paper-mulching. The advantage of paper-mulching in DM yield was closely corresponded with positive gain in plant height and tiller density through improvement in light penetration [14] and prevention of plant damage from weeds [15]. Positive effect of paper-mulching on DM yield matched with several crops such as paddy rice [8], lettuce [6], tomato [7] and turmeric [16].

Weed control management by sowing setaria had no significant ($P > 0.05$) effect to suppress weed DM yield in Trial-2 (data not shown). However, 1 g/m^2 DM production from setaria reduced 4 g/m^2 of DM production from weeds ($y = 104.553 - 0.247x$, $r = 0.52$, $P > 0.05$). In several stand establishments of perennial species, annual species are used to suppress weeds and mitigate the competition of crops from weeds. In the present study, annual setaria has characteristics to give good growth in early summer and suppress summer weeds [13].

Neither IVDMD nor CP content was affected by any weed control in either trial. Consistently positive effect of any weed control practice on quality attributes was hardly obtained in either trial, while decline in CP content under weeding and paper-mulching practices was common at the second and first defoliation in Trial 1 and 2, respectively (Table 2). Living mulch with white clover improved plant nutrition by enhancing phosphorus uptake in maize [9]. The present paper-mulching and hand-weeding could not contribute to herbage quality of DL napiergrass, possibly due to the negative correlation of DM yield with quality attributes under similar fertilization in this species. Dwarf napiergrass cv. Mott, which has almost equivalent plant attributes to DL napiergrass, had IVDMD and CP content at 67.5% and 13.2%, respectively [17], almost corresponded with the present IVDMD at 74% and 60% at the first and second defoliation, respectively, in Trial 1.

4. Conclusions

Paper mulch is not common to use in DL napiergrass cultivation, while it proved to be effective to avoid weed damage and facilitate good growth with

high DM yield of this species. Cost of paper mulch is 50 yen/m (Sanyo Seishi Co. Ltd., Tottori) and setting of paper mulch is a labor-extensive weed control practice. Thus, based on the amount of natural seed bank of weeds, paper mulch or other degradable mulching material can be applied to DL napiergrass, so as to reduce weed competition at the establishment. Annual setaria-sowing gave advantage to get herbage yield at the first defoliation of DL napiergrass, although prompt harvest time of annual setaria should be examined in the mixed cropping with DL napiergrass.

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Cytogenetic Analysis of Three Species of *Astyanax* Genus (Pisces, Characidae, *Incertae sedis*) from Freshwaters of Upper Paraguay Basin, Mato Grosso State, Brazil

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Abstract: Cytogenetic studies have shown that the genus *Astyanax* has extensive karyotypic variability among species and many are the works developed about this group in different river basins in Brazil. The Tangará da Serra area (Mato Grosso State, Brazil), which contains tributaries of both Amazon and Upper Paraguay River basins is, therefore, a natural watershed. Thus, in this work, the karyotypes of three species of *Astyanax* from the Sepotuba River's tributary streams were analyzed by using conventional methods of chromosome analysis. *Astyanax marionae* presented 2*n* =48(12m+10sm+12st+14a) and *NF* (fundamental number) = 82 with evident heterochromatin in several chromosomal regions. *Astyanax* cf. *asuncionensis* presented 2 *n* = 50 (20m + 12sm + 6st + 12a), *NF* = 94 chromosomes and with evident heterochromatin in few chromosomes and Ag-NOR in one chromosome pair. *Astyanax* cp. *scabripinnis* presented 2 *n* = 50 (12m + 10sm + 10st + 18a), *NF* = 82 with evident heterochromatin at most of the chromosomes in pericentromeric region and a strongly marked block on a telocentric chromosome pair. The Ag-NORs were observed near the heterochromatic region. The karyotypic differences found, probably occurred by chromosomal rearrangements, resulting in a heterogeneous model of chromosomal evolution who probably involved both Robertsonian chromosomal rearrangements to *A. marionae*, as not Robertsonian to other *Astyanax* species. This indicates that although these species share the same hydrographic basin (Upper Paraguay) are isolated in different microbasins and the absence in the gene flow may have allowed the establishment of independent rearrangements in each population.

Key words: Chromosome, fish, karyotype, Pantanal, Robertsonian rearrangements.

1. Introduction

The genus *Astyanax* Baird and Girard, 1854 has wide distribution comprising over one hundred species and subspecies in Neotropical rivers [1, 2]. It is one of the most widely distributed taxa of freshwater fish in the Americas, occurring from Argentina through the Mexican border with the United States [3] being dominant in South America [4].

In Brazil, fish of this genus are popularly known as "lambaris" or "piabas". They inhabit various environments including the headwaters of rivers and streams [5, 6]. The genus *Astyanax* was part of the subfamily Tetragonopterinae [6] and now it is allocated among several *incertae sedis* genus within Characidae. This occurs because during the period in which most of the Characidae subfamilies were described, only global similarities between the species were considered and origin of each character was not important, which contributed to a dubious characterization of several groups [7].

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Cytogenetic studies have shown that the genus *Astyanax* has extensive karyotypic variability among species. This includes variation in the diploid number [8-13, among others] concerning the number and positioning of nucleolar organizer regions (Ag-NORs) and heterochromatin [9, 14-21, among others], and the presence of B chromosomes as in *Astyanax scabripinnis* [22-33, among others].

These studies indicate that the chromosomal diversity presented by the genus *Astyanax* is very broad, presenting different karyotypes among the specimens of distinct basins, among specimens of the same basin in different streams, among isolated populations of the same stream and between sympatric populations. This shows a remarkable process of the phenotypic and karyotypic variability in this group of fish, making it a very interesting group of organisms for evolutionary biology studies. In the present study, the karyotypes of three species of *Astyanax* from headwater streams of the Upper Paraguay Basin were analyzed, with the use of conventional methods of chromosome analysis.

2. Materials and Methods

The karyotypic analysis were done with three species of *Astyanax*: 5 specimens (2 males, 3 females) of *Astyanax* cf. *asuncionensis* Géry, 1972 collected in the Dimba's stream (14°39'46.86"S, 57°44'35.82"W), 14 specimens (10 males, 4 females) of *Astyanax marionae* Eigenmann, 1,911 collected in the region of Salto Maciel waterfalls (14°41′34.92″S 57°48′13.81″W) and 8 specimens (6 males, 2 females) of *Astyanax* cp. *scabripinnis* (sensu Eigenmann, 1927) collected in São José stream (14°33′48.89″S 57°24′32.51″W) (Figs. 1a-c). All species were collected in the Upper Paraguay basin in tributary streams of the left bank of the river Sepotuba as well as the river itself (Salto Maciel waterfall), of Tangará da Serra, Mato Grosso State, Brazil (Fig. 1d). Voucher specimens of different species of *Astyanax* studied were deposited at the Laboratory of Animal Cytogenetics, Universidade Federal de Mato Grosso (*A.* cf. *asuncionensis* = 5,379 − 5,383; *A. marionae* = 5,377, 5,378, 5,387 − 5,397, 5,482; *A.* cp. *Scabripinnis* = 5,431, 5,443 − 5,449).

The mitotic metaphase chromosomes were obtained from kidney cells to study in fish [34], with some adjustments. The C-band technique was used for analysis of constitutive heterochromatin [35]. The Ag-NORs were detected by staining of Silver Nitrate ($AgNO_3$) [36]. The identification of chromosomes was performed according to the criterion of ratio arms (RB) [37].

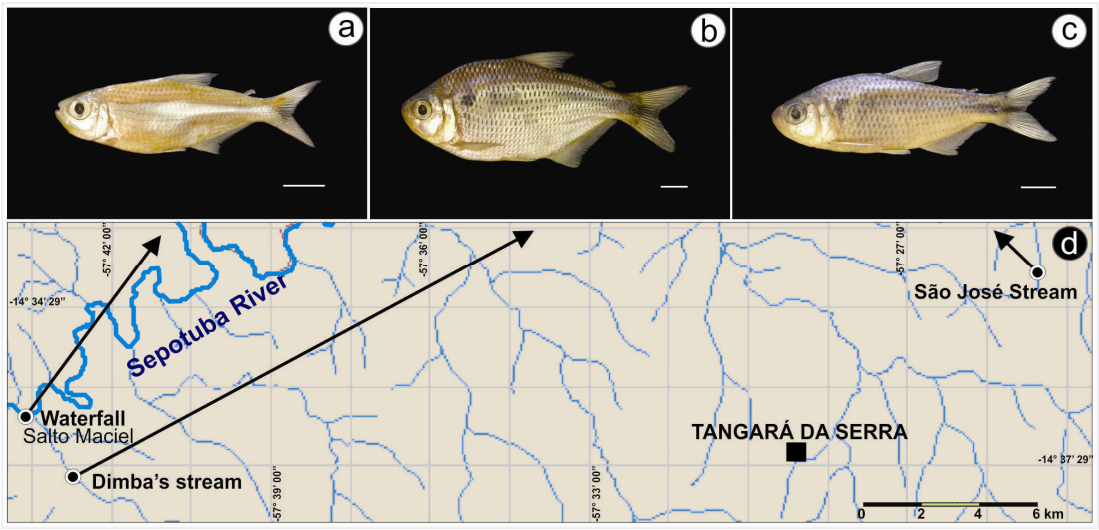

Fig. 1 (a) *Astyanax marionae*; (b) *Astyanax* cf. *asuncionensis*; (c) *Astyanax* cp. *Scabripinnis*; (d) Map showing the areas where each studied species were collected. Source: Ministério do Meio Ambiente (Mapa Interativo I^{3}Geo–Version 4.0)—with adaptations. Bar: 1 cm.

3. Results

Astyanax marionae presented modal diploid number of 2 *n* = 48 chromosomes (12m + 10sm + 12st + 14a) and *NF* (fundamental number) = 82 (Fig. 2a). Constitutive heterochromatin was observed in the pericentromeric region on the short arms of metacentric and submetacentric chromosomes, in the centromeric region of some acrocentric chromosomes and telomeric regions of subtelocentric chromosomes (Fig. 2b). The Nucleolus Organizer Regions (Ag-NORs) analyzed are present in a pair of subtelocentric chromosomes adjacent to the marking of heterochromatic region (Fig. 2c).

Astyanax cf. *asuncionensis* presented modal diploid number of 2 *n* = 50 chromosomes and a karyotypic formula equal to (20m + 12sm + 6st + 12a) and *NF* = 94 (Fig. 2d). Constitutive heterochromatin not was evident in most chromosomes of metaphases analyzed (Fig. 2e). The Ag-NORs were observed in a pair of chromosomes (Fig. 2f). *Astyanax* cp. *scabripinnis* presented modal diploid number of 2 *n* = 50 chromosomes (12m + 10sm + 10st + 18a) and *NF*= 82 (Fig. 2g). Constitutive heterochromatin was observed in most chromosomes in the pericentromeric region, showing a heterochromatic block most evident in one chromosome pair (Fig. 2h). The Ag-NORs coincide with the heterochromatic regions present at the telomeric region of a chromosome pair (Fig. 2).

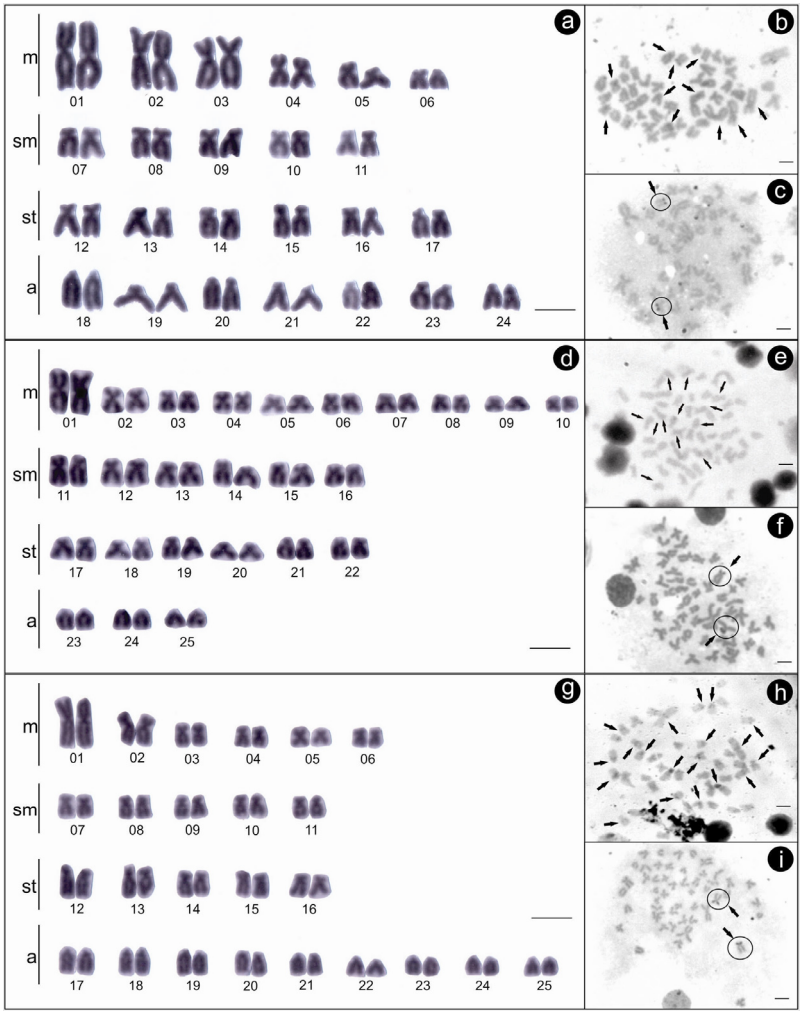

Fig. 2 Chromosomal macrostructure of *Astyanax* species studied and information on karyotype, heterochromatin and Ag-NORs, respectively. *Astyanax marionae* (a, b, c), *Astyanax* cf. *asuncionensis* (d, e, f), and *Astyanax* cp. *scabripinnis* (g, h, i). Bar: 5 μm.

4. Discussion

Cytogenetic studies in natural populations of freshwater fish from the Neotropical region have provided important information about the chromosomal changes that occur in some species, possibly favored by geographical isolation. Even the size of the hydrographic system may be an important factor, as long as it allows these species to evolve through geographic isolation in headwater tributaries, where the barriers between the population may be physical, chemical or even biotic [14, 15]. This demonstrates the importance of researches, for example, in the Upper Paraguay River Basin, especially in areas of headwater streams, where there are still few studies with *Astyanax* species.

It was observed that among the three species studied (*A. marionae*, *A.* cf. *asuncionensis* and *A.* cp. *scabripinnis*), two showed the same diploid number (2 *n* = 50). This result is the most frequent in species of the genus *Astyanax*, as it has been described for *A. bimaculatus* [38, 39], *A. mexicanus* [40, 41], *A. eigenmanniorum* [26], *A. scabripinnis* [16, 22, among others], *A. altiparanae* [19, 42, 43], *A. giton*, *A. parahybae* [11], *A. bockmanni* [44], an endemic species of *Astyanax* [45], *A. jacuhiensis* [46], *A.* aff. *bimaculatus* [39], *A. laticeps* [13], *A. ribeirae* [47] among others.

Most species of *Astyanax* have 2 *n* = 50 chromosomes, and if we consider other groups *insertae sedis* in Characidae, but with relative proximity of *Astanax* as *Juiaba* or *Brycon* which also have 2 *n* = 50, we see that this can really be a plesiomorphic condition. Thus, different values of 2 *n* = 50 may be apomorphic, as it is observed in *A. marionae* which has 2 *n* = 48 chromosomes. Another interesting fact is that this species has morphological characteristics similar to *A. fasciatus* with red caudal fin, besides the karyotypic similarities.

Another notable event in the karyotype of *A. marionae* is that the first, second and third pairs of metacentric chromosomes are the largest of the karyotype, with little difference in size between them. To explain this fact, we can say the modal diploid value 2 *n* = 48 may have been originated by a centric fusion between smaller chromosomes (acrocentrics) resulting in another large metacentric pair and to explain the third large pair, there may have been translocations from other chromosomes or a second idea would be a duplication *in tandem* in a pair of metacentric chromosomes. Anyway, it is a different karyotype and it is certainly synapomorphic in relation to other *Astyanax*, as already observed by others authors to *A. fasciatus* [48].

Astyanax cp. *scabripinnis*, is possibly a new species within the complex *scabripinnis* once that the karyotype of 2 *n* = 12m + 10sm + 10st + 18a, is very different from those found in other populations of this species complex of other basins [8, 17, 22, 23, 50-56, among others]. This hypothesis suggest a trend in maintaining the number of pairs of meta and submetacentric chromosomes in three and four respectively, and possibly is a plesiomorphic feature [22]. Thus, the result of this population could be a synapomorphic condition in relation to that found in other locations. As the group has a high rate of speciation, and being this population apart from the others found in the country, as in this case, it is natural and expected that it would be different from the others.

Astyanax asuncionensis showed a less diversified karyotype with 2 *n* = 50 chromosomes as found in most species that were once called *A. bimaculatus*, although the first metacentric pair is not so much bigger than the rest of the complement.

5. Conclusions

Considering the results of this paper, it can be said that the *Astyanax* found in streams from the Upper Paraguay Basin, Tangará da Serra, Mato Grosso State, show differences when compared with results from other studied areas. So this is a promising region for

cytogenetic analysis because it can increase the knowledge of different groups and collaborate with phylogenetic and evolutionary analysis through research with other *Astyanax* species unstudied and also studies with other groups of fish that occur in this hydrographic basin.

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Anesthetic Management during Cesarean Section in English Bulldogs

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Abstract: The authors describe their experience with the management of the perioperative period, general anesthesia and the postoperative period in English bulldogs undergoing elective cesarean section and its effect on the neonates. The anesthetist for animals undergoing cesarean operation must be aware of any special needs, not only of the patient undergoing surgery, but also of the neonates. Anesthetic drugs administered to the pregnant patient will readily cross the placenta and affect them, with the exception of local anesthetics. Pregnant female patients are at increased anesthetic risk due to pregnancy-associated physiological alterations, such as altered pulmonary function. The anesthetist is often called to perform anesthesia on brachycephalic dogs for an elective cesarean section. Due to their conformation, these animals may have one or more anatomical abnormalities of the upper airways, which compromise the ability to ventilate adequately. The induction and recovery phases of anesthesia can be extremely dangerous in these patients, but the maintenance phase is generally fairly straightforward because the airways are controlled during maintenance. In addition, vagal tone is frequently high and this can contribute towards significant bradycardia and further airway narrowing. All these reasons make general anesthesia in brachycephalic dogs undergoing cesarean section rather complicated.

Key words: Anesthesia, cesarean section, English bulldog, brachycephalic syndrome.

1. Introduction

The English bulldog is a breed with various health issues, such as brachycephalic syndrome, heart disease and orthopedic, skin and reproductive problems. The reproductive management of English bulldog bitches involves various different skills that include artificial insemination, ultrasound monitoring, and the performing of a cesarean section at the end of the pregnancy. Brachycephalic airway syndrome refers to a particular set of upper airway abnormalities which affects brachycephalic dogs. The upper airway abnormalities occurring in this syndrome include stenotic nares, an elongated soft palate, a hypoplastic trachea and everted laryngeal saccules [1]. Any of these upper airway abnormalities can cause increased airway resistance to airflow and the work of breathing, respiratory distress, stridor, reduced exercise tolerance and, in more severe cases, cyanosis and collapse [2].

In addition, stress-induced and pain-induced tachypnea, with subsequent increased work of breathing, will cause a significant increase in negative pressure in the airways and this negative pressure can exacerbate airway obstruction [3]. Stress, excitement and pain can all cause increased sympathetic nervous system activity resulting in vasoconstriction and increased systemic vascular resistance [4].

In particular, advanced pregnancy greatly increases the workload on the heart, mainly when the patient is in dorsal recumbency during surgery and the pressure of the abdominal organs on the diaphragm causes hypoventilation, hypoxia, and hypercapnia further compromising respiratory function [5]. In addition, there are many consequences due to an increase of intra abdominal pressure. Cardiac output and stroke

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volume after a short initial rise due to the squeezing of the blood from the abdominal veins is then compromised through decreased venous return, elevated intra-thoracic pressure, and increased systemic vascular resistance, resulting in decreased cardiac preload, increased cardiac afterload, and depressed ventricular function [6]. The fetus is vulnerable to changes in the mother's cardiovascular system due to poor autoregulation of fetal blood flow and the uterine perfusion being pressure-dependent.

Therefore, it is of fundamental importance to take into account all the physiological changes which occur during pregnancy and which affect the anesthetic management of the patient: decrease in functional lung residual capacity and increase in minute ventilation, increase in cardiac output and blood volume, delayed gastric emptying with decreased esophageal sphincter tone. Anesthesia can cause further complications as anesthetic drugs and equipment can exacerbate or even cause airway difficulties and respiratory compromise [7]. Anesthetic drugs, dehydration and intraoperative fluid losses will result in maternal hypotension and pregnant animals appear to have blunted cardiovascular responses and are less able to tolerate hypovolemia [8].

During the evaluation and before anesthesia, the patient should be handled quietly and gently to avoid stress-induced tachypnea with subsequent increased work of breathing and possible further respiratory dysfunction. Tranquilization is often necessary to keep thepatient calm, and analgesia is required for any patients in pain [3]. Although it is essential that the patient should receive adequate anesthetic agents to provide immobilization and analgesia for the surgery, it is advisable to use minimal doses as those agents depress respiration in puppies. In fact, it is not possible to selectively anesthetize the mother since anesthetic drugs readily cross the placenta and affect the neonates [9]. During surgery, pregnant animals have a reduced need for inhalant agents due to the sedative effect of circulating progesterone and elevated β-endorphins levels [10].

The aim of this study was to identify the critical points in the management of the perioperative period, general anesthesia and the postoperative period in English bulldogs undergoing elective cesarean section considering both the characteristics of this breed and the physiological changes that occur during pregnancy. The authors describe their experience, suggesting basic steps to decrease maternal anxiety, avoid hypoxia and hypovolemia, provide analgesia, reduce anesthetic requirements and ensure smooth induction and recovery.

2. Materials and Methods

Eight English bulldog bitches underwent elective cesarean section at the Veterinary Teaching Hospital of the School of Veterinary Medical Sciences, University of Camerino. The dams were given a thorough examination with particular attention to cardiopulmonary function and hydration status. As part of the examination, an ultrasound examination was performed to establish viability of the fetuses and fetal anomalies, such as hydrops fetalis. A blood sample was drawn from the dam to perform hematocrit, total protein, glucose, calcium, and blood urea nitrogen evaluation. An x-ray of the abdomen was taken to assess the presence of gas and the amount of food inside the stomach. The bitches were then taken into the premedication room. The patients were administered 2 μg/kg of dexmedetomidine (Dexdomitor 0.5 mg/mL, Orion Pharma Janssen Animal Health, Italy) and 0.2 mg/kg of butorphanol (Dolorex 0.1 mg/kg, Intervet, Italy) intramuscularly and were preoxygenated from 2 min to 5 min by face mask while the sedatives were taking effect. Delivery of 100% oxygen allows the functional residual capacity to fill with oxygen. During this time, an intravenous catheter was inserted. After adequate sedation, only two bitches were suitable for epidural anesthesia. This was performed with a single injection

of 1 mL/6 kg of lidocaine (Lidocaine 2%, Fort Dodge Animal Health, Italy) via the lumbosacral (L7-S1) intervertebral space using a 22-20 gauge spinal needle. The total volume of the epidural solutions was made up to 0.2 mL/kg BW with sterile saline. The animals were placed in sternal recumbency with their legs pulled forward. To confirm the correct placement of the needle, the loss of resistance test was performed using a syringe filled with fluid connected to the needle. The drugs were injected over a two-min period. The patients were induced to anesthesia slowly with 2-3 mg/kg of propofol (Rapinovet 10 mg/mL, Intervet, Italy) intravenously and intubated as soon as this took effect. Once intubated, the endotracheal tube cuff was carefully inflated. The abdomen was clipped, the initial surgical preparation of the skin was carried out and oxygen administered. The patients were transferred to the operating theatre, where the surgeon was ready with scalpel blade in hand. The anesthesia was maintained with isoflurane vaporized in oxygen delivered through a circle breathing system and spontaneous ventilation. The operating table was inclined in reverse Trendelenburg position to decrease the weight of the pregnant uterus on the diaphragm. A final abdominal scrub was performed and surgery was under way. Ringer's lactate solution (RL solution, Fresenius Kabi, Italy) was infused IV at 10 mL/kg per hour, or more depending on the blood pressure, using a peristaltic infusion pump (B Braun Compact). A multi-parameter monitor (BeneView T8, Mindray, China) was used to record the following physiological variables: respiratory rate, (RR), heart rate (HR), electrocardiogram (ECG), non-invasive systolic, diastolic and mean pressures (SAP, DAP, MAP), oxygen saturation of hemoglobin (SpO_2), esophageal temperature (T), end-tidal isoflurane (E`Iso) and carbon dioxide concentration (PE`Iso), inspired oxygen concentration. E`Iso was allowed to reach 1.5% and the vaporizer setting was decreased by 0.2% every 5 min until the depth of anesthesia was deemed sufficient (assessment of palpebral reflex, mandibular tone, absence of response to surgical stimulus and changes in cardiovascular parameters). The puppies were delivered as rapidly as possible and turned over to assistants. The care of the newborn puppies consisted in clearing the pharynx of mucus and fluid, vigorous rubbing, oxygen delivery by face mask, gentle chest compressions, swinging neonates in head-down position and sublingual naloxone and/or atipamezole if the pups were slow to begin vocalizing. To remove the mucus and fluids from the nostrils, a nasal aspirator—commonly applied in human neonates—was utilized as described by Goericke-Pesh and Wehrend [11].

Once breathing appeared adequate, the neonates were placed in a thermic incubator. After surgery, the dams were observed closely. The endotracheal tube was left in place as long as possible until the patient began to chew and oxygen was delivered. Once the tube was removed, the animal's head and neck were extended. Ventilatory function was monitored for at least 1 h following recovery from anesthesia.

Statistical analysis was performed with one-way analyses of variance (ANOVA) for repeated measures to evaluate the differences between two bitches that had received epidural lidocaine and those that had not. Data are reported as mean ± standard deviation (SD) values. Differences were considered significant when $P < 0.05$.

3. Results

Blood test values were normal in all animals included in the study although a mild anemia was evident, probably due to an increase in the total blood volume resulting in the red blood cells not keeping pace with plasma expansion. No correlation was observed between the severity of the anemia and the number of puppies [12].

The depth of sedation was sufficient for the application of a venous catheter and to administer oxygen without causing further stress to the animals, with the exception of one, where the administration of

an additional dose of dexmedetomidine at 0.5 μg/kg IV proved necessary to deepen the level of sedation. The dams that received epidural lidocaine were calm enough to allow this procedure to be carried out.

After induction, the animals were rapidly intubated to prevent gastric reflux caused by gastric emptying delayed as a result of increased intra-abdominal pressure. Moon et al. reported that 5 out of 9 bitches whose death was associated with caesarean section had pneumonia, suggesting that aspiration is also an important risk factor in dogs [13]. There were no significant differences in total propofol dose (2.2 ± 0.4 mg/kg) in the animals included in the study. Oxygen delivery was prolonged after intubation and was suspended when the dams were transferred to surgery to avoid fetal hypoxia and acidemia due to the transient apnea that can occur following propofol administration.

The anesthesia was maintained with isoflurane in spontaneous ventilation. In one case, manual ventilation proved necessary. To decrease the degree of atelectasis, the pop-off valve on the anesthetic circuit was closed and the reservoir bag briefly squeezed at a pressure of 15-20 cm H_2O. During general anesthesia Ringer's lactate solution was infused IV at 10 mL/kg per hour, proving sufficient to support the cardiovascular system and to compensate for the substantial fluid losses associated with surgery. In only one case, a constant rate infusion of dobutamine (5 μg/kg/min) was required to increase hypotension (< 60 mm/Hg) that was refractory to simply increasing the rate of fluid administration and decreasing anesthetic delivery. The bitches that received epidural anesthesia required less anesthetic volatile agents (E`Iso 1.4 ± 0.12) but the difference was not statistically significant compared to dogs that had not received it (E`Iso 1.5 ± 0.10). There were no differences in the other parameters recorded.

No dogs required rescue analgesia in the perioperative period. Isoflurane was discontinued at the end of surgery and the dogs were transported to the recovery room. Surgery was carried out without complications in all cases and all dogs were discharged from the hospital 3 h later. The mean (± SD) surgery time, from the skin incision to the last stitch, was 40 ± 7 min. In one case, both re-induction and re-intubation proved necessary due to dyspnea and cyanosis during the postoperative period. In no case did the dogs require rescue analgesia or show signs of urinary retention during the postoperative period. Apart from two litters, where the neonates died following severe edema, two puppies showed slight signs of reduced respiratory and cardiovascular function, but the situation was rapidly taken care of. In these cases, one drop of atipamezole—the specific antagonist of dexmedetomidine—was injected into the root of the tongue. No puppies required intubation. Forty-eight puppies were born overall.

4. Discussion

The veterinarian anesthetist is always faced with the dilemma of having to anesthetize the mother, who may already be compromised, without adversely affecting the fetus. All drugs have direct and indirect effects on the puppies, but the veterinarian must be familiar with the anesthetic technique and have a good knowledge of the specific problems concerning each breed.

Although the $alpha_2$ agonists should be avoided as a premedication agent in parturient patients, the use of a new and safer drug at very low doses, such as dexmedetomidine, did not result in adverse side-effects. In addition, the effects of $alfa_2$ agonists can be reversed by specific antagonists whose dosage should be based on the amount of agonist administered initially and the time that has elapsed since the agonist was administered. Dexmedetomidine is an $alpha_2$ adrenoreceptor agonist with reported synergistic sedation and analgesia with opioids [14, 15]. Dexmedetomidine is the dextro-rotatory isomer of medetomidine and may have some advantages over the racemate in terms of increased analgesic potency.

In addition, although administration of dexmedetomidine still causes bradycardia, this may be less severe than with racemic medetomidine [16]. A perceived advantage of dexmedetomidine sedation is the availability of an antagonist, atipamezole, which produces rapid recovery from sedation. In this study, the use of low doses of dexmedetomidine was not associated with preoperative and perioperative adverse effects, such as bradycardia, bradypnea or hypotension. It might, therefore, be useful as a premedication drug prior to cesarean section, providing excellent sedation without evident depression in puppies. In fact, only two puppies out of 48 were in need of sublingual drops of atipamezole.

All opioids cross the placenta and can cause neonatal respiratory depression, but their effects can be reversed by antagonist agents such as naloxone. Furthermore, the degree of opioid-mediated respiratory depression is generally minimal and easily controlled, especially if the patient is going on to anesthesia and will be intubated and maintained on 100% oxygen. Butorphanol is a synthetic kappa opioid agonist and mu opioid antagonist and has been used extensively in a wide variety of veterinary species [17]. The use of this drug alone promotes minimal changes in cardiopulmonary function and the respiratory depression caused by this opioid is less than that induced by morphine [18]. Butorphanol has been used for obstetric analgesia because it induces less respiratory depression and provides moderate levels of sedation [19]. It was included in our protocol without any adverse effects, providing excellent analgesia and probably allowing a reduction in the total dose of propofol required for induction.

Propofol, an alkyl phenol hypnotic, has been studied as an intravenous anesthetic in dogs and it was reported to be a short-acting, rapidly metabolized agent, characterized by a virtual lack of any cumulative effects and by rapid recovery [20]. The use of propofol to produce narcosis resulted in increased newborn survival rates and in good vitality following surgery with minimal residual fetal depression [21]. In this study, slow administration (from 30 seconds to a minute) of propofol prevented the onset of apnea and induced minimal changes in heart rate. After induction, the animals were rapidly intubated and oxygen delivery was prolonged after intubation. The evaluation of oxygen saturation indicated the adequacy of ventilation.

The lumbosacral epidural anesthesia is noted for its simplicity, safety and effectiveness, and is one of the most frequently used regional anesthetic techniques described for surgical procedures which are caudal to the umbilicus [22]. Epidural anesthesia is frequently recommended for cesarean section because, unlike other anesthetic techniques, it does not depress the puppies. In this study, only two dams received epidural anesthesia because the premedication allowed adequate sedation, but if the dam will not cooperate for epidural anesthesia, this procedure should be avoided. A reduced volume of 2% lidocaine (1 mL/6 kg) was satisfactory for epidural anesthesia maybe due to the distension of the epidural veins, which decreased the size of the epidural space and increased the spread of the local anesthetic. Furthermore, chronic exposure to progesterone altered the permeability of the intercellular connective-tissue matrix facilitating diffusion through the nerve [23]. Epidural anesthesia, using a local anesthetic, increases the risk of hypotension following sympathetic nerve blockade. Hypotension induced by epidural anesthesia can be managed with crystalloid solutions and dobutamine in continuous rate infusion to prevent decreased uterine perfusion and fetal compromise. During surgery, in this study, the only dog that presented hypotension was one of those that had not received epidural anesthesia. It is likely that the hypotension occurred following uterus exteriorization from the abdomen. In this case, the uterus was of considerable size. The hypotension was treated with 5 μg/kg/min of dobutamine in constant rate infusion.

For the duration of the surgical procedure, the

patient should be maintained on the lowest possible concentration of inhalant anesthetic agents, bearing in mind that anesthetic gas requirements are believed to be reduced in the pregnant animal. E`Iso was maintained between 1.4 ± 0.12 and 1.5 ± 0.10, without any significant differences between the dogs that received epidural anesthesia and those that had not. We believe that this could either be due to the fact that the number of animals that received additional epidural anesthetic lidocaine was too small to show any significant differences between the two groups or that the dose of epidural lidocaine might have been too small to provide supplemental analgesia.

No animal presented hypothermia probably due to the short duration of the surgery (40 ± 7 min).

In conclusion, elective procedures should be considered in Bulldogs which constitute 17% of all cesarean sections [13]. In an elective situation a complete physical examination and blood tests would be performed. Any period of hypoxemia in the mother is also a period of hypoxemia in the fetus and this can significantly decrease fetal viability at birth. Therefore, the dams should be preoxygenated by face mask during the preoperative period. General anesthesia should be induced quickly. Oxygenation and ventilation are more easily controlled and the airway is protected with endotracheal tube placement. The anesthetist should choose the anesthetic protocol that will have minimal effects on the puppies and the one with which they are most familiar. During anesthesia a multiparametric monitor should be used to record vital parameters and in particular pulse oximeters should be used to evaluate oxygen saturation and capnometers to indicate the adequacy of ventilation. Maternal hypoventilation can lead to fetal hypoxia and either manual or mechanical intermittent positive pressure ventilation may be required. The operating table should be inclined in reverse Trendelenburg position to decrease the weight of the pregnant uterus on the diaphragm and ensure greater respiratory excursion. Recovery can often be the most critical phase of anesthesia in patients with airway disease and dysfunction, so monitoring and support should continue into the recovery period. Of fundamental importance for the success of a caesarean section, is the perfect coordination between anesthetist, surgeon and support staff and the minimization of excitement and stress for the dogs in the perioperative and recovery period.

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Patella rustica Linnaeus, 1758 (Gastropoda, Patellogastropoda) Inhabiting Coast of Skikda (Algérie)

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Abstract: The study was undergone on the distribution of abundance of *Patella rustica* Linnaeus, 1758, with the objective of evaluating of its abundance along the rocky shores of the coast and located sites with high density. A total of twelve sampling sites were selected along the gulf of Skikda. Each station was sampled using quadrats of 25 cm^2. In the present study, two environmental parameters were measured "*in situ*" in the water column at each sampling station (water surface temperature, pH). Biotic communities of the rocky shores are considered to be generally controlled by physical factors such as temperature and desiccation. Therefore, three measurements were made for each abiotic parameter during this survey and mean values were used for statistical analysis. Consequently, the data of abundance (expressed in frequency) of species were calculated based on the distributed individuals along the quadrat. *P. rustica* is distributed mainly in the East more than in the West particularly from S1 to S5. The condition index calculated at 12 stations revealed important seasonal variations, with the maximum during summer season highlighting three stations (S2, S11 and S12). This index is also very important in S3 and S4. The factors of pollution at these points had not affected the abundance and index condition.

Key words: Abundance, index of condition, environmental parameters, *Patella rustica*.

1. Introduction

Algerian coast is generally SW-NE direction; it extends from Marsat Ben M'Hidi West (North West of Tlemcen) to Cape Roux East (Annaba) 1,280 km (Fig. 1). It appears as a succession of bays and more or less open bays separated by very steep terrain. Skikda is located on the coast of the Mediterranean Sea (North Eastern Algeria), and it's bordered on the West Jijel and Annaba on the East. The city is an important industrial area, with chemical factories, refineries, thermal power plants, marble industry with three ports (ancient port, new port of hydrocarbons transportation and fishing port). Coastal marine ecosystems are influenced by heavy anthropogenic pressure due to the location of human settlements. The main sources of pollutants in coastal waters are domestic, industrial and port wastes [1].

Limpets are common rocky intertidal grazers and mostly belong to the family Patellidae Rafinesque, 1815 (Gastropoda: Patellogastropoda). The revision of Christiaens (1973) reduced the 240 species described for the genus *Patella* (sensu lato) to 32 [2]. The Family Patellidae comprises four extant genera *Patella*, *Cymbula*, *Helcion* and *Scutellastra* and in the Mediterranean Sea, only two genera, *Cymbula* and *Patella*, are recognized [3]. *P. caerulea* Linnaeus, *P. rustica* Linnaeus and *P. aspera* Lamarck are the most common Mediterranean species of the genus *Patella*, *P. caerulea* is an endemic Mediterranean species, *P. aspera* and *P. rustica* inhabit both the Mediterranean and Atlantic coasts [4]. *P. rustica* is an intertidal rocky shore Patellogastropd limpet and was first described

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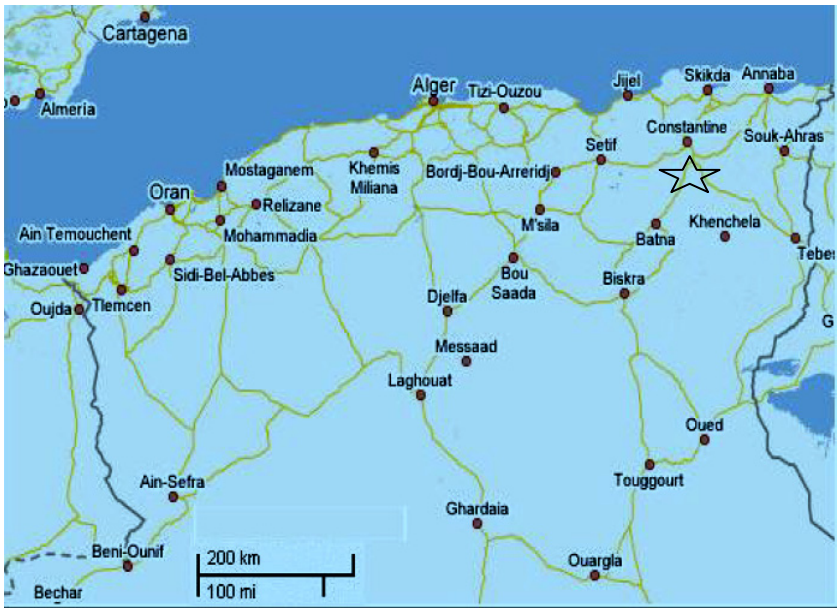


Fig. 1 Locations of study region (Skikda: North East Algeria) along the Occidental Mediterranean sea between Annaba and Jijel (Google map, 2008). Algerian coastline extends over approximately 1,300 km. ☆: Working area.

by Linnaeus 1758. This genus has been subdivided into two subgenera: *Patella s.s*. and *Patellastra* [5-7]. This division was based on the observation that all the species of the first subgenus have pluricuspid radular teeth with three cusps, while species of the second subgenus have pluricuspid teeth with only two cusps. On the basis of this criterion, *P. aspera* and *P. caerulea* have been ascribed to the first subgenus and *P. rustica* to the second [6, 7]. As regards, karyotypic structure of both *P. rustica* and *P. caerulea* have a haploid complement of $n = 9$ whereas *P. aspera* has only haploid complement of $n = 8$) [7]. Concerning the geographic distribution of *P. rustica*, some authors reported that it's restricted on Mediterranean Sea and on the Atlantic coasts from Biarritz (France) to Mauritania and it is also available in the Cape Verde Islands, Canary Islands, Selvagens, Desertas and Madeira, but not sampled in the Azores [8, 9]. Moreover some authors indicated that the form of *P. rustica* determined in the Macaronesian Islands is a different species *P. piperata* Gould, 1846 [8].

P. rustica, *P. caerulea* and *P. aspera* are common limpets on Mediterranean rocky shores where they are sited on the upper and mid costal rocky shores, low inter-tidal zone and the sublittoral fringe, respectively [10]. During grazing periods, *P. rustica* moves up to the supralittoral [4, 7, 11].

This species is easily recognized by a very high symmetrical cone, with a rounded base [12] and morphological characters on the shell (the characteristic black spots that adorn its shell near the apex [4, 13] (Fig. 4). Regarding Bulleri et al. (2000), the feeding consists mainly on macrophytes eukaryotic filamentous [14]. Limpets of the genus *Patella* (L.) play a keystone role in the structuring of littoral communities and their grazing strongly influences algal composition and diversity [8].

There is no available data conducted such benthic fauna along Skikda rocky coastline. Only many studies on the benthic macrofauna of sedimentary substrates were conducted [15]. These data are fulfilled in Algerian base data (Bental) what aims to assess the species richness and taxonomic structure of the macrofauna of soft substrates benthos of the Algerian coast. Several studies were carried out on the faunal inventory and especially bottoms of the ports and open areas (bays and gulfs) of the Algerian coast [15].

The main objective of this present study was to

evaluate (1) how the total abundance and condition index of this limpet varied from site to site in each season collected from twelve localities on rocky shore of Skikda coast and (2) how two types of physical factors (pH and temperature) affect the patterns fluctuations of Abundance and condition index.

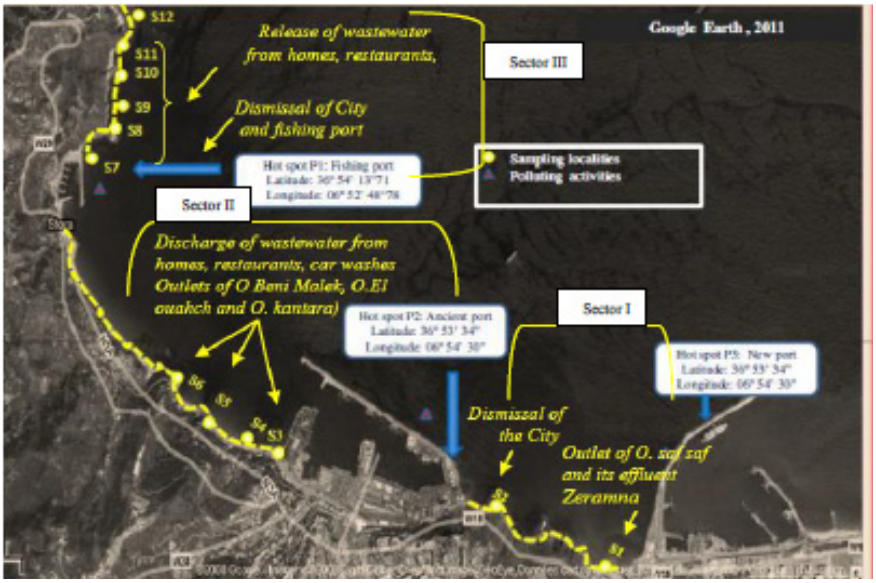
The species studied is *Patella* (*Patellastra*) *rustica* Linnaeus 1758 (= *P. lusitanica* Gmelin 1791).

2. Material and Methods

2.1 Sampling Stations

Between September 2008 and August 2009, the study was undertaken in the coastal zone from the petrochemical complex (36°55'N) to the beach ravin des lions (36°52'N) to investigate a spatial variation limpet (*P. rustica*) on rocky shore. The choice of 12 stations was made taking into account many pollution sources (domestic, industrial and agricultural), accessibility and abundance of limpets. The working stations are located on a transect of a discontinuous length of approximately 5.48 km from the coast as follows: beach Saf Saf (S1), Goat isle (S2), Horses beach (S3), Château Vert beach (S4), Marquette beach (S5), Military beach (S6), fishing port of Stora (S7), mollo beach (S8) Miramar beach (S9), a creek near Miramar (S10), the career beach (S11) and the beach of the ravin des lions (S12) (Fig. 2). These locations are wave-exposed rocky, they are representative of coastal conditions of the Bay and are influenced by different types of anthropogenic activities.

These included sites affected by industrial activities (S1, S2), sites influenced by urban waters (S2, S3, S4, S5, S6, S8) and sites near to the port (S1, S3, S7), four sites as bathing area seem to be away from known sources of pollution that were as used as reference sites (S9, S10, S11 and S12). Survey of total hydrocarbon and heavy metal in the coastal waters and sediment of Skikda indicated a significant pollution



(P1) Fishing port of Stora (P2) Ancient port (P3) New port

Fig. 2 Map of Skikda gulf showing the working stations divided in three sectors.

Sector I: Saf Saf beach (S1), Isle Station (S2); Sector II: Horses beach (S3), château vert beach (S4), Marquette beach (S5), Military beach (S6); Sector III: Rear fishing port of Stora (S7), Mollo beach (S8), Miramare beach (S9), small creek near Miramare beach (S10), Carrière beach (S11) and Ravin des lions beach (S12).

Table 1 Heavy metal content in mg/g in the surface sediments of the main ports of Skikda [19].

Ports	Mercury	Cadmium	Lead	Copper	Zinc	Chromium
Ancient port	18	0.12	30	65	170	40
New port	3.3	1.58	120	200	770	70
Algerian standards	1.5	3	250	150	500	250

Table 2 Variable characters of external morphology of soft parts in *P. rustica* used for identification [5].

Characters	*P. rustica*
Head	Grey
Cephalic tentacles	Dark grey
Buccal disc	Cream
Foot	
Sole color	Dark grey or blue with edge and centre cream
Shape	Circular
Pallial tentacles	
Pigment	Translucent
Arrangement	2 series of different lengths usually alternating

Table 3 Summary of environmental characteristics in gulf of Skikda (Mean ± SD are presented over study period).

Parameters	Minimum	Maximum	Mean ± standard deviation	Algerian standard (JORA, 2006)
pH	6.89	8.43	8.08 ± 0.26	6.5-8.5
T, °C	10.60	30.6	18.27 ± 4.41	30

but contamination by hydrocarbons discharge is more important [16-18]. Metallic pollution previously reported in sediments and waters nearby or at the selected areas is shown in Table 3.

Urban pollution from the city of Skikda, pollution from the industrial zone, maritime traffic and port activity are responsible for the metal contamination of sediments from the old port and the new port. High concentrations of essential metals (copper, zinc, manganese) are detected in the old port. A spike in mercury 13.09 mg/g with an average of 7.24 ± 4.47 mg/g was recorded in the new port, probably due to contamination by sewage inputs and maritime traffic. Mercury and zinc at high levels in the sediments of Skikda Gulf. This contamination source for the rivers (Oued Saf Saf and Mahsen), which are collectors of all pollutants and marble unit (Enamarbre), located at the industrial zone and wastewater units chemical industries located on the coastal fringe [17, 18].

2.2 Sampling

To examine geographic variation in abundance and condition index quarterly, specimens are handpicked in the morning at the time of low tides from the stations showed on Fig. 2. Determination was based on shell morphology [5, 9, 12] and other characters were used, including the color of the foot, and the internal texture of the tentacles (Fig. 3 and Table 2).

At each station, the authors selected the most representative shore. The height started from 0.7 m tidal level until 1.5 m. Each station was sampled using quadrats of 25 cm^2. In whole area, the presence /absence of *P. rustica* was recorded three times for each unit during seasonal monitoring.

Specimens of *P. rustica* were washed with water and brushed. The condition index was calculated each season for each station on ten individuals selected to be as close as possible sizes (class 18.1-22.6 mm). The formula for calculating the index AFNOR [20] was adopted. The total mass includes the shell, the soft tissues and water mantle.

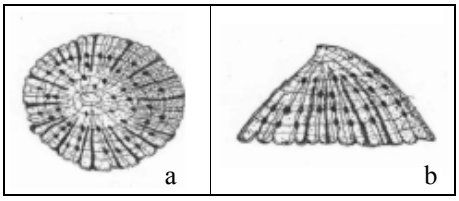


Fig. 3 Top (central) (a) and lateral (left) (b) views of *P. rustica* [12].

$$C = \frac{STW(g)}{TW(g)} \quad (1)$$

STW: Soft tissue weight (g); *TW*: Total weight.

The surface seawater temperatures and pH were recorded at the same time *in situ* at each working area using a multiparameter WTW 340i and mean values were used for statistical analysis.

The data of abundance (expressed in frequency percentages) of limpet species were calculated based on the presence/absence in 25 units distributed along the 1.5 m vertical transect. The abundance and condition index were calculated for each station. Mean values and standard deviation were calculated of the 12 stations. One way analyses of variance (ANOVA) were used to test for differences in species abundance among working localities and seasons. A significance level of $P < 0.05$ was carried out for all statistical tests and data were transformed, log10(X + l), prior to analyses. All statistical analyses were performed using Minitab 14.0 and SPSS17.0.

3. Results and Discussions

3.1 Environmental Variables

The general trend of the 2 environmental variables throughout the bay is shown in Table 2. Temperatures and pH were within the optimal range. The Algerian standards are between 6.5 and 8.5 [21]. The extreme values of pH are 6.89 to 8.43 (Table 2). The highest mean value is 8.38 ± 0.04 at station S12 whereas the minimal mean is registered at stations S2 (7.47 ± 0.10). The magnitude of water surface temperatures was significant (10.6 °C-30.6 °C). These results are in agreement with previous studies conducted in Skikda bay [17, 18].

Over the study period, temperature didn't fluctuate much among the sampling locations (Fig. 4) but the temperature was significantly different during four seasons (One-Way ANOVA analysis, $P < 0.001$) (Fig. 6). The lowest temperature was recorded at station S12 (winter: 10.60 °C) while the highest was at station S8 (summer: 30.6 °C). The pH fluctuates slightly among the study stations.

The pH value showed differences between stations ($P < 0.001$) and among seasons ($P < 0.05$). The highest value was registered at Station S12 (summer: 8.43) while the lowest was measured at station S2 (summer: 6.89). It was noted that the stations located close to the petrochemical complex (S1 and S2), ancient port (S3) located East of Stora fishing port (S7), recorded pH close to neutrality. The other stations were tending to basic throughout the study period.

The sea surface temperatures (SST) registered in autumn (18.25 ± 1.05 °C) is higher than the spring (16.66 ± 0.83 °C). In winter and summer, the temperatures recorded were respectively 13.04 ± 0.83 °C and 24.87 ± 1.25 °C. The temperature of the air was highest in summer (25.50 ± 2.08 °C) and lowest in winter (12.4 ± 0.79 °C. And therefore the temperatures of water of all stations have shown that seasonal periods were related to weather conditions. It's Mediterranean-type climate.

Mollo beach (S8) had the highest mean value of temperature over the study compared to other stations (26.44 ± 2.11 °C) in summer. The latter is a bathing beach frequented by summer visitors and characterized by multiple domestic waste waters from restaurants and habitations.

Patella species are known as keystone because of their important influence on the structure and function of the rocky shore communities [22]. The disposal of urban and industrial discharges directly into the sea through outfalls proved one of the main causes of the deterioration of the coastal ecosystem in Algeria generating malfunctions of various physical and biological compartments of this ecosystem [17]. The temperatures of surface water on the twelve stations were highest in summer and lowest in winter. The pH found range from neutral to alkaline.

Orton (1920) and Olive (1995) expose that Sea temperature is considered one of the most influential factors in controlling breeding in marine invertebrates [26].

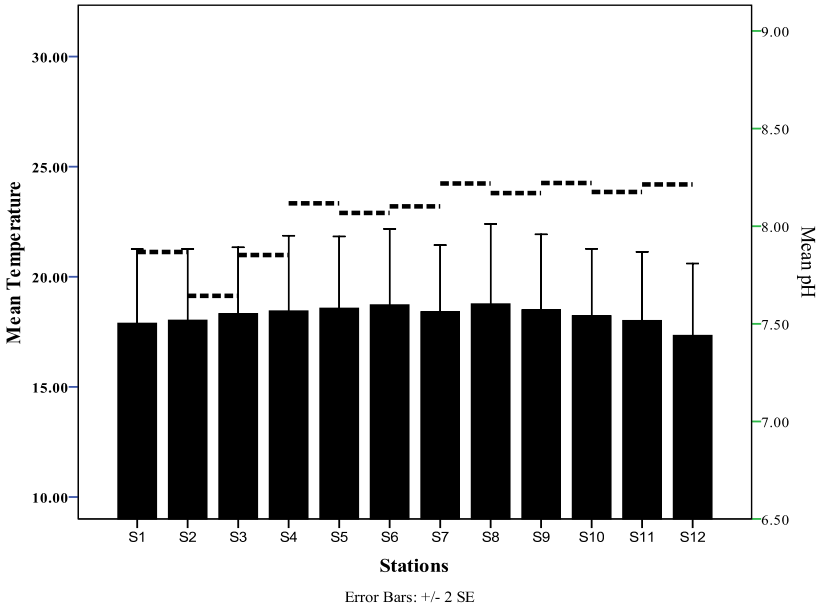


Fig. 4 Mean (+1 SE) temperature and pH measured on each station in gulf of Skikda over study period. Three measurements were taken at each station during 4 seasons.

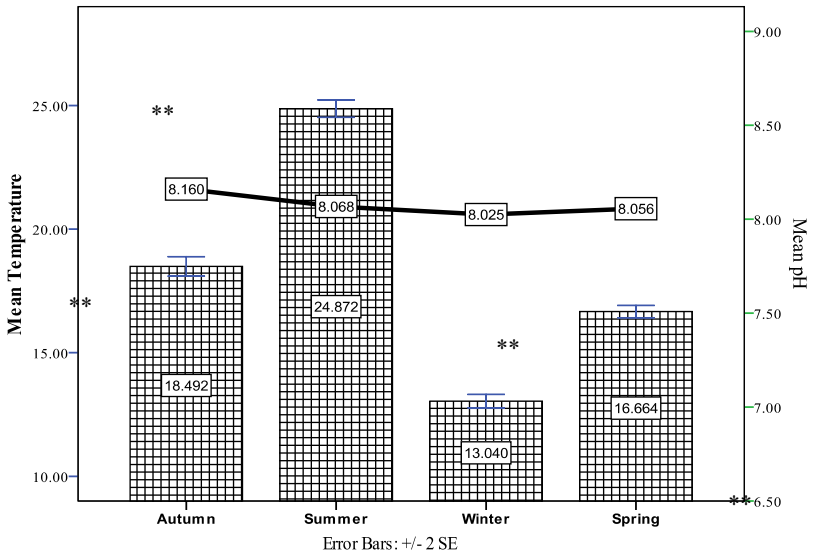


Fig. 5 Mean (+1 SE) temperature and pH measured on each season in gulf of Skikda over study period. Three measurements were taken at each station (3×4 measurements per season).

(a) (b) (c) (d)

Fig. 6 Working stations: A: Colony of Barnales (Station S6); B: *P. rustica* and others limpets (S4); C: Measurements of *P. rustica in situ* (S2); D: colony of *P. rustica* (Station S2).

Vermeij (1971) mentions that body temperatures of limpets are higher than those of other intertidal mollusks [23]. Limpets are probably more prone to heating than other organisms. Howsoever only few published studies are available for *P. rustica* Linnaeus, 1758 on this warm-water species [24].

3.2 Distribution of Fauna and Algae

During this monitoring, several species of algae and macrofauna have also been found (Fig. 6). For example the most common and dominant algae intertidal algae (*Ulva lactuca* Linné, 1753; *Ulva rigida* C.Agardh 1823; *Enteromorpha compressa* (Linné) Nees, 1820; *Enteromorpha intestinalis* (Linné) Nees, 1820; *Cladophora rupestris* (L) Kützing, 1843; *Chaetomorpha capillaris* (Kützing) Boergesen 1925) are the species coating the rock surface in the stations S1 to S5. Others algae are inventoried only at western sector (S3) such as *Ralfsia verrucosa* (Areschoug) Areschoug 1845; *Scytosiphon lomentaria* (Lyngbye) Link 1833; *Nemoderma tingitanum* Scousboe ex Bornet, 1892; *Porphyra leucosticta* Thuret in Le Jolis 1863 and *Verrucaria amphibia* Clemente, 1814).

Our results indicated communities of crustaceans (*Pachygrapsus marmoratus* Fabricius, 1787; *Chthamalus stellatus* Poli, 1795; *Chthamalus montagui* Southward, 1976; *Euraphia depressa* (Poli, 1791); *Pollicipes pollicipes* Gmelin, 1789; *Ligia italica* Fabricius, 1798) and molluscs (*Gibbula racketti* Payraudeau, B.-C., 1826; *Gibbula pennanti* Philippi 1836; *Littorina punctata* Gmelin, 1791; *Littorina neritoides* Linné, 1758; *Murex* (*Hexaplex*) *trunculus* Linné, 1758; *Monodonta turbinata* Born, 1780; *Diodora graeca* Linné, 1758; *Acmaea unicolor* Forbes,1844. Many others limpets were found such as *Siphonaria pectina* Linné, 1758 (air-breathing sea snail or false limpet); *Patella vulgata* Linné, 1758; *Patella caerulea* Linné, 1758; *Patella ferruginea* Gmelin, 1791; *Patella rustica* Linné, 1758; *Patella aspera* Röding, 1798; *Patella nigra da Costa*, 1771 and *Emarginula sicula* J. E. Gray, 1825.

3.3 Limpet Spatio-Temporal Distribution

The percentage of abundance is listed in Table 2. Data of abundance (expressed with the number of specimens (N)) of species *P. rustica* was calculated based on the presence/absence in the three quadrat of 25×25 square cm found along the 1.5 meters vertical transect. The abundance assessments made from September 2008 to August 2009 clearly showed that the number of individuals in this sampling area is increasing at the East.

In the present study, we have considered only the true limpet *P. rustica*. In this area, the spatial distribution of *P. rustica* within Skikda bay was relatively uniform. A total of 385 individuals of true limpet *P. rustica* were collected from twelve stations. This species was found at all stations. Of these, analyses were undertaken on seasonality and spatial distribution. *P. rustica* was found numerically dominant at four stations respectively S2, S4, S1, S3 and S12 (Table 4, Fig. 6). The abundance was relatively low at S8, S9, and then significant increases occurred at S10 and S11 and S12. Individual's species patterns of *P. rustica* reached maximum abundance especially in summer and autumn. Globally, the main distributional characteristics indicated two sectors with low and high abundance. This zone (S1) has relatively high total abundance, it's could be could be linked to the nature smooth of rocky shore that may provide habitat to this limpet. Moreover *P. rustica* is generally associated with steep surfaces found in the upper eulittoral of exposed rocky shores [25]. The results were significantly higher in the S2 (15.06 ± 2.25%) and S4 (13.51 ± 2.21%) than in other working areas. The limpet *P. rustica* was very scarce in sampling (2.08 ± 0.73%) at S8 (mollo beach).

Mean abundance was significantly greater in the sectors I and II. On The West sector (III) also high higher numbers of limpets were recorded on stations S10 and S11 (Fig. 7). Significant effects of station and season ($P < 0.001$) detected in the density of *P. rustica*

(Table 5). In addition the interaction station x season was not significant (one way Anova; $P > 0.05$), indicating that the density was not changing in all stations over the seasons. The Turkey HSD test classified sites into the following ($P < 0.05$): S8, S9 S7, S6, S11, S10, S5, S12, S3, S1, S4 and S2 and sequenced seasons as winter, spring autumn and summer.

P. rustica has found plenty on, the Eastern part of the bay during the period of survey. In three stations (S7, S8 and S9), numbers of limpets were relatively low compared with the all study sites this is probably due to human impacts and domestic waters. Analysis of abundance patterns indicated a fewer limpets in winter and spring over the assessment in all localities (respectively 8.83% and 19.48%). Further these patterns showed also a high number of specimens predominantly at stations S2 and S4 during the sampling survey (Table 3). These stations are respectively at 0.78 km and 1.25 km West of hydrocarbons harbor, then S3 is adjacent to ancient harbor and S4 is on about 3.34 km from new port and 0.3 km from S3.

The densities has relatively contracted too few from S6 to S9 to rise again (S10-S12), and other species of limpets are recorded especially *P. caerulea* and *Siphonaria pectinata* in the middle and west of the bay.

The highest abundances were found mostly in the Eastern of the bay (Fig. 7) and during summer and

Table 4 Mean (± SD) abundance (%) of *P. rustica* in the present study along twelve stations (2 transects per stations).

Stations	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
Abundance (%)	10.65 ± 1.43	15.06 ± 2.25	10.13 ± 1.58	13.51 ± 2.21	8.83 ± 1.09	6.49 ± 2.52	4.68 ± 1.62	2.08 ± 0.73	3.64 ± 0.62	9.09 ± 1.91	6.49 ± 1.07	9.35 ± 1.37

Table 5 Results of a one-way fixed factor ANOVA testing the abundance between site and period and the interaction (site and seasonality).

	F (site)	F (season)	F (site*season)
Abundance	10.548**	47.901**	1.240^{NS}
P value	0.000	0.000	0.245

* $P < 0.05$; ** $P < 0.001$; ^{NS} $P > 0.05$.

DF (site) = 11; DF (season) = 3; DF (site*season) = 33.

Displayed are degrees of freedom (DF), F-ratios (F), and corresponding significance levels (*P*).

R Squared = 0.963 (Adjusted R Squared = 0.926).

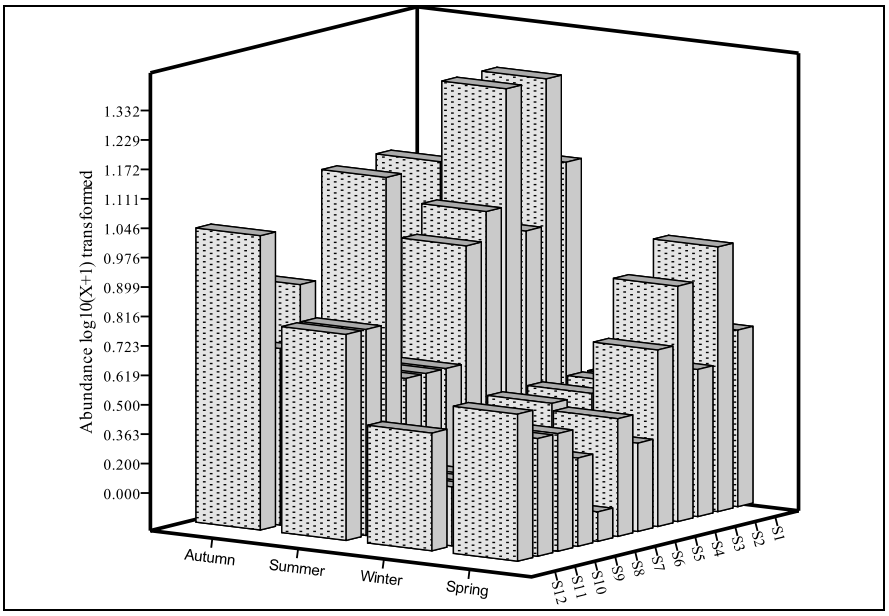


Fig. 7 Seasonal pattern of *P. rustica* with the frequency (%): Abundance $\log_{10}(X+1)$ transformed.

autumn periods which coincide with the periods of high temperatures. The total abundance (individuals/m^2) indicated a large difference in magnitude between the lowest (individuals/m^2 for Mollo beach) and the highest (individuals/m^2 at station S2 in July). The abundance of this species was also recorded at S3 and S4 with other limpets particularly *P. caerulea* L., and *Siphonaria pectinata* (Gastropoda, Pulmonata).

3.4 Condition Index

Parallel to the study of population abundance, showed a variation of the condition indices of limpets between stations and seasons. This is due to several factors including the stage of reproduction (gametogenesis activities) and nutrition. The condition index is the physiological state of the animal in relation to the environmental conditions (increase or decrease in the availability of food) and reproductive status (weight gain or loss related to the gametogenesis). Thereby condition index (C.I.) is an indicator of the physiological status of the true limpets. This index is an ecophysiological measure of the health status of the animals that summarizes their physiological activity (growth, reproduction, secretion etc.) under given environmental conditions. During the period of sexual dormancy, this quotient is a good indicator of mollusk growth [7]. It was clear from the study period that the high value is recorded on summer especially when condition index was maximum followed by autumn. Limpets from site S2 recorded higher condition index than the other sites over the study period. This is probably due to the high value organic matter (domestic waste) noted in previous studies [16, 18]. In our study, the high indices are measured during summer (between 30 June and 10 July) and mid-autumn (between 10 September and 15 October) seasons; the low indices are shown at mid-winter (between 15 January and 7 February) and spring (between 21 March and 8 April). Mostly high condition indices correspond to repining period and lowest indices coincide with release of eggs. The condition index was variable during the seasons, with a pronounced peak in the summer at Ravin des lions beach (32.30% ± 6.65%). The lowest mean condition indices of limpets were those from Mollo beach and horse beach in winter (respectively 14.75% ± 0.07% and 16.06% ± 1.49%). On the Portuguese coast, it was reported that gametogenesis began in June, sexual maturity increased until September and October when spawning (the emission of gametes) took place by December to January [26]. From the Basque coast of Spain, Othaitz, (1994) indicated multiples spawning events and in the Mediterranean Sea, *P. rustica* has long breeding period with less synchronous multiples spawning occurring between August and November [4]. *P. rustica* showed a peak in gonadic relative weight during autumn and spawn mostly in December-March, and individual spawning probably occurs only once a year [11].

Globally, condition index varied between 25.97% ± 4.86% to 16.71 ± 2.42%. Important values were measured in limpets issued from S2, S11 and S12 (Fig. 8). At station S12, limpets showed particularly high indices of condition (32.30% ± 6.65%) marked in summer while minima (14.60 ± 1.56%) were recorded in winter in limpets sampled from station S9 (Miramar beach). On the whole, the indices were higher in autumn and summer than in spring/winter. This difference revealed a filling of the shells of limpets from S2, S11 and S12. This difference can due to seasonal change and reproduction cycle. The comparison between seasons showed a single annual maximum, focused in summer (mid-July and August). Seasonal variations were highly important with maxima in summer (25.09% ± 4.29%) followed by the autumn indices (23.25% ± 4.37%). The condition indices in winter and spring were respectively 17.65% ± 2.20% and 21.51% ± 2.91%.

A one-way ANOVA was used to compare mean differences in C.I among limpets, indicated that there

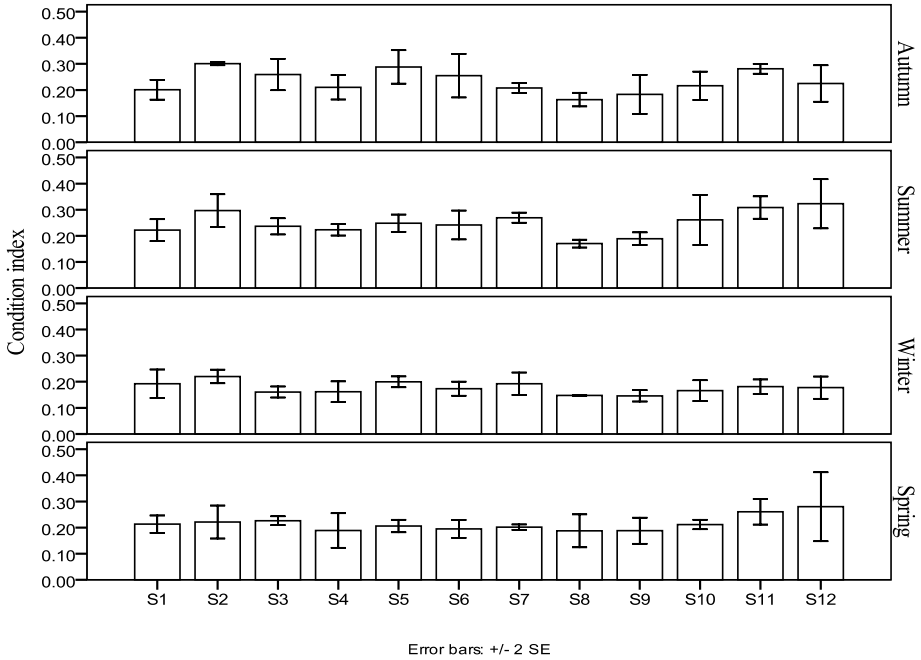


Fig. 8 Seasonal fluctuations in the mean (+1 SE) condition index (CI) of limpets from 12 working locations over study period from September 2008 to August 2009. Results of one-way ANOVAs contrasts between stations and seasons ($P < 0.001$).

Table 6 Results of a one-way fixed factor ANOVA testing the condition index between site and period and the interaction (site and seasonality).

	F (site)	F (season)	F (site*season)
Condition index	6.056^{**}	20.584**	0.888^{NS}
P value	0.000	0.000	0.636

* $P < 0.05$; ** $P < 0.001$; ^{NS} $P > 0.05$.

DF (site) = 11; DF (season) = 3; DF (site*season) = 33.

Displayed are degrees of freedom (DF), mean square errors (ms), F-ratios (F), and corresponding significance levels (*P*). $R^2 = 0.998$ (Adjusted $R^2 = 0.997$).

are significant differences in the CI values among limpets from 12 locations (Table 6). The Tukey HSD test classified sites into the following ($P < 0.05$): S8, S9, S4, S1, S10, S6, S7, S3, S5, S12, S11 and S2 and seasons winter, spring, autumn and summer.

3.5 Environmental Factors (T °C and pH) on Limpet Distribution and Condition Index

In order to see the relation between the physicochemical factors and limpet's abundance, we used Pearson's correlation matrix based on seasonally values (Table 7).

Abundance was positively correlated with sea temperature (*r*-value = 0.586, $P < 0.01$, Table 7). No significant correlations were observed between abundance and pH. However, significant correlations were measured between abundance and condition index (*r*-values = 0.498, $P < 0.01$).

Condition index showed a positive correlation with temperature (*r*-values = 0.479, $P < 0.01$). This suggests that the high abundance and condition index of true limpet *P. rustica* were induced by temperature increase.

No relationships were apparent between pH and the sea surface temperature. The value of pH was circumneutral in S1, S2 and S3 but pH values at the remaining stations (From S4 to S12) had alkaline values. In this study, the evolution of this parameter is very slight.

Table 7 Pearson's correlation matrix of biological and environmental parameters. The *r*-values shown in this table indicate statistical significance when *P*-values are < 0.01. Cross indicates that *r*-values are not significant. Correlation coefficients between Abundance (Abun), Condition index (CI), sea surface temperature (SST) and pH.

		Abun	CI	SST	pH
Abun	Pearson correlation	1	0.498	0.586	x
CI	Pearson correlation	0.498	1	0.479	x
SST	Pearson correlation	0.586	0.479	1	x
pH	Pearson correlation	x	x	x	1

Correlation is significant at the 0.01 level (2-tailed).

Some studies showed that temperature may have direct effects by killing limpets, and important sublethal effects, such as influencing metabolic rates and feeding rates [23]. For Orton (1920), Sea temperature has long been considered one of the most influential factors in controlling breeding in marine invertebrates in [19] and biotic communities of the rocky shores are considered to be generally controlled by physical factors such as temperature and desiccation [27].

Body temperature is strongly influenced by ambient conditions and equilibrates with water temperature soon after the limpet is submerged. Consequently low-shore limpets that are exposed for shorter periods may have mean body temperatures up to 14 °C less than high-shore individuals (Davies, 1970 in [23]. Bonner et al. (1993) quoted that motility of limpet was reduced at pH 5.5 submerged but once returned to normal sea-water individuals recovered. And more at pH 2.5, total inhibition of movement occurred and when returned to normal sea-water half had died [28].

The variations in environmental factors such as temperature, and pH among sites would influence the physiological state of the animals. The surface temperature of the water could be a major determinant of reproductive success and thus opportunities for dispersal of *P. rustica* [25].

Abundance and condition index of *P. rustica* during this survey were correlated with temperature (respectively: r = 0.586; *P* = 0.00 and r = 0.594; *P* = 0.00). This joins the work reported that the air temperature and sea, monitor especially periods of reproduction (autumn) and recruitment and these are the main factors in the distribution of *P. rustica* on the Atlantic Coast, and initial growth (winter) periods [25]. Also this author noted the regions characterized by a warmer climate such as the Southern Portuguese coast and the Bay of Biscay was correctly modeled as having high densities of *P. rustica*. This study showed a strong correlation between spatial distribution and condition index of *P. rustica* and the water temperature measured in these sites (Table 7).

4. Conclusions

Thompson et al. (2002) reported that intertidal communities are open ecosystems, with steep environmental gradients and their susceptibility to both terrestrial and marine disturbances makes them more vulnerable than sublittoral and offshore habitats) in [29]. Pollution effects are most pronounced in enclosed bay, harbors and estuaries [22].

The composition of sessile communities is particularly useful as a baseline for ecological monitoring because such organisms are unable to avoid disturbances in the marine environment and thus, the composition of the community reflects their common history (Fa et al., 2002) in Ref. [29].

Our results show that *P. rustica* nearly always extend from S2 to S6. Density was a sensitive marker of delimitation of these stations, because nearly a quarter of the population is located within two sectors S1 and SII.

In conclusion *P. rustica* varied both in density and condition index. These variations may be due to the geography of stations and partially attributed to abiotic factors and floral resources. We must add that

monitoring the seasonal variations of environmental factors and limpet abundance in Skikda bay between September 2008 and August 2009 has augured insights into the interactions of biological and physiochemical factors. Sea surface temperatures mainly affected the seasonal variation in abundance and growth of rocky shore true limpet. Until now no study was undergone on limpets (*Patella* spp.) throughout the rocky coast of North East Algerian coast. Few studies are undertaken on the Oran coast on *Patella caerulea* [22]. Thereby, we have focused on this species of true limpet (*P. rustica*) because it is a relatively sedentary and widespread distribution and abundant and easily identified while *P. caerulea* was also plentiful notwithstanding it is hard to identify (variation in morphology and may be confused with *P. aspera*). Moreover, it had been used in some areas in the Mediterranean Sea and near such harbors as Italy, Spain, Turkey and Portuguese coast [9, 11, 31-33]. All these data for the gulf of Skikda showed the same trend: a high ecological quality status for this species, also in the stations affected by anthropogenic activities. So this benthic macrofauna thus appears as a good indicator of changes in the environment [35]. The density and condition index of limpets was steady in overall between locations. The most sampling stations located on Skikda coast are polluted by coastal water pollution by slopping a lot of contaminants such as industry and domestic pollutants. However, the stations from S9 to S12 are uncontaminated zone in terms of industry. Several authors [16-18] have shown increasing pollution across the Skikda coastline especially in the areas of quite considerable number of industrial plants (Eastern bay), that are at the origin of disturbances and increased pollution as well as municipal dismissals and three main port activities on the bay [17, 35].

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Serum Protein Capillary Electrophoretic Pattern in Camels (*Camelus dromedarius*): Influence of Age and Sex

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Abstract: The objective of this study was to characterise serum protein capillary electrophoretic pattern in relation to the age and sex in dromedary camels. Fourteen healthy young camels (age: 3-5 months), 12 adult male and 10 female camels (age: 5-8 years) were used. Blood samples collected from the jugular vein were used for the determination of serum proteins by capillary electrophoresis technique. Female camels had significantly ($P < 0.05$) higher serum-[Protein] of 63.7 ± 6.6 g/L (reference range = 51-74 g/L) compared to the other age groups. Adult male camels showed significantly ($P < 0.05$) higher percentage of albumin fraction (60%) compared to the other age groups. The concentrations of α1 and α2 globulin fractions were significantly ($P < 0.05$) higher mean value in young camels compared to the other groups (3.5% and 8.5%, respectively). β-globulin fraction was not affected significantly by the age. The concentration of γ-globulin fraction (26%) in lactating camels was higher ($P < 0.05$) compared to the other age groups. Significantly ($P < 0.05$) A/G ratio was observed in young camels. Sex had no significant effect on serum protein fractions. The results obtained were compared and interpreted in the light of finding reported by other investigators in camels, humans and other animals.

Key words: Age, camels, serum protein capillary electrophoresis, reference values.

1. Introduction

Serum proteins are known to comprise about 6-7 g/dL of the plasma [1]. The plasma proteins are involved in nutrition, maintenance of osmotic pressure, buffering acid-base balance, transport of smaller ions and molecules, haemostasis and protective effect of the immune proteins [1]. Many of these plasma protein change markedly in diseases [2, 3] and with age [4, 5].

Capillary electrophoresis of serum proteins (CE) is an established and effective method which has been used as a screening tool for the clinical diagnosis of many diseases in humans [6, 7] and animals [8]. Normal serum proteins electrophoretic pattern is composed of five fraction, albumin, α_1-globulin, α_2 globulin, β-globulin and γ- globulin [1]. Therefore, the clinical interpretation of CE is based on the variation in the content of one or more of these five major fractions. However, species differences between the animals have been observed by Keay et al. [4].

Therefore, the aim of the study was to validate the use of CE in camels and to determine the normal serum protein capillary electrophoretic pattern in relation to the age and sex.

2. Materials and Methods

The animals used in this study were selected from the herd of the camel research unit of the University of Khartoum. Animals were maintained on grazing and browsing trees and shrubs in the vicinity of the camel

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unit and occasionally received concentrate supplements. Blood samples were collected form fourteen healthy young camels (7 males and 7 females, age: 3-5 months) and 22 adult camels (12 male and 10 female, age: 5-8 years) by jugular venipuncture using plastic syringes (7.5 mL, Pirmvetta®, Laboratory Technique, GmbH, Germany). The blood samples were centrifuged and haemolysis-free serum samples were harvested in sterile containers and frozen at -20 °C. The fractionation of serum proteins was determined using a capillary electrophoresis technique utilising a biochemical analyser (Roche Hitachi Modular, Roche).

Statistical analysis was performed by using SPSS for Windows version 17.0. The distribution of the individual data was determined by using a One-Sample Kolmogorov-Smimow adjustment test. The statistical measurements of serum total protein fraction were estimated by using descriptive statistics procedures of the same programme. ANOVA tests (Levine's Test and Post Hoc Test) were used to assess the possible significant differences between the age groups. The mean difference was considered significant at $P \leq 0.05$.

3. Results

Fig. 1 shows the normal pattern of CE in dromedary camels. The pattern of CE identified one albumin, two α-globulin (α_1 and α_2), one β-globulin and one γ-globulin fractions.

The mean values of serum total protein and CE fractions are shown in Table 1. The mean value of serum total protein [Protein] obtained for female camels, 63.7 ± 6.6 g/L (reference range: 51-74 g/L) was significantly ($P < 0.05$) higher compared to the other age groups. Adult male camels showed highly significantly ($P < 0.0001$) higher percentage of albumin fraction (60%) compared to the other age groups. The α_1 and α_2 globulin fractions values (3.5% and 8.5%, respectively) were significantly ($P < 0.01$) higher in young camels compared to the other groups. The β-globulin fraction was not affected significantly by the age. Lactating female camels showed a significantly ($P < 0.01$) higher mean value of γ-globulin fraction (26%) compared to the other age groups. The lactating females showed the lowest A/G ratio that was significantly ($P < 0.001$) different from other groups. Sex had no significant effect on serum protein fraction.

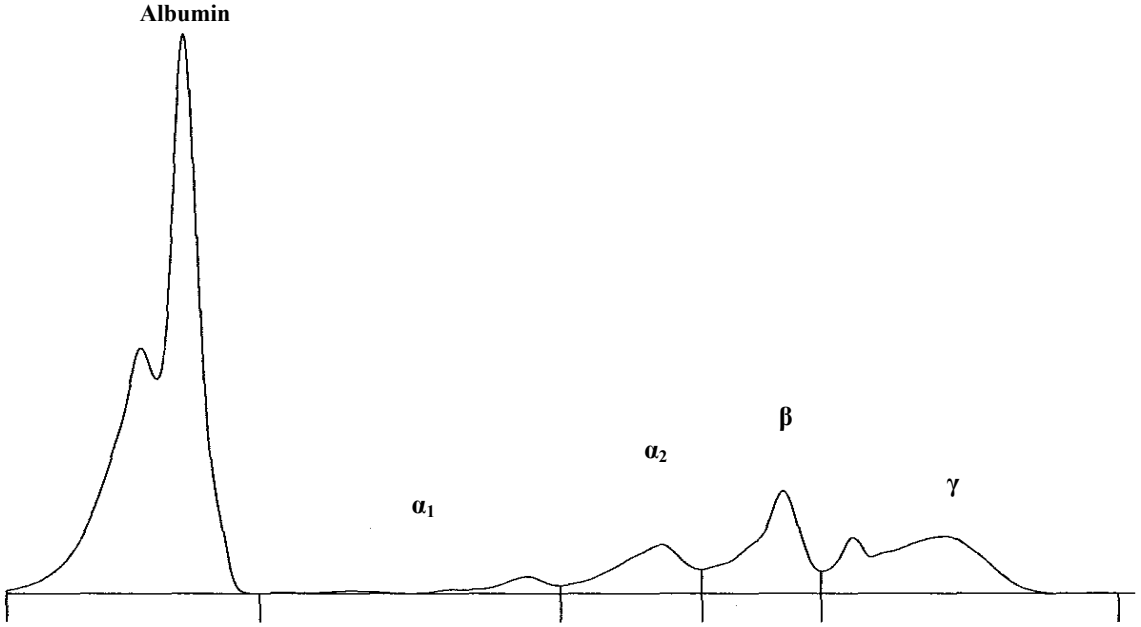

Fig. 1 Representative serum protein capillary electrophoresis pattern of healthy camels (*Camelus dromedarius*) of various ages and sex ($n = 36$).

Table 1 Mean values (± s.d.)* and range of serum-[Protein] (g/L) and serum protein capillary electrophoresis fractions (%) of healthy camels (*Camelus dromedarius*) in relation to age and sex (*n* = 36).

	Young male and female	Lactating females	Adult males
Number of animals	14	10	12
Age	3-5 months	5-8 years	5-8 years
Serum-[Protein] (g/L)	54.8 ± 4^a 50-62	63.7 ± 6.6^b 51-74	57.7 ± 4.3^a 51-65
Albumin fraction (%)	58 ± 4.7^a 52-67	50 ± 4.9^b 39-54	60 ± 2.2^a 57-63
α_1-globulin fraction (%)	3.5 ± 0.5^b 2.6-4.4	2.6 ± 0.4^a 1.9-3.3	2.7 ± 0.6^a 1.8-3.9
α_2-globulin fraction (%)	10 ± 1^a 8.5-12.6	9.4 ± 1^b 7.8-10.9	8.6 ± 1^b 7.5-11
β-globulin fraction (%)	10.4 ± 0.8^a 9-12	11.7 ± 1.7^a 8.5-14	10.9 ± 0.7^a 9.9-12
γ-globulin fraction (%)	17.7 ± 5.2^a 9-25	26.3 ± 5.2^b 23-39.5	17.7 ± 1.7^a 15.2-21
A/G ratio	1.4 ± 0.3^a 1.1-2	1 ± 0.2^b 0.6-1.2	1.5 ± 0.1^a 1.3-1.7

* m ± s.d. × 1.96 indicated the lower and the upper limits, Brackets ([ ]) donate concentration, s.d. = standard deviation.

[a, b] Means within the same row bearing different superscripts are significantly different at $P \leq 0.05$.

4. Discussion

This study validated the use of capillary electrophoresis technique for the first time for fractionation of serum proteins in dromedary camels. The results also provided critical evaluation of the effects of age and sex on the electrophoretic pattern of serum proteins. The CE depicted in Fig. 1 illustrates five peaks comprising one albumin, α_1 and α_2, β and γ-globulins fractions (Fig. 1). However, Chaudhary et al. [5] have reported that serum protein electrophoresis on agarose gel in camels produced six peaks comprising one albumin, α_1 and α_2, β_1 and β_2 and γ-globulin fractions. The variation in the serum electrophoresis pattern between the present study and the study conducted by Chaudhary et al. [5] may be due to differences in methodology used.

The reference range of serum-[Protein] obtained in the present study for adult camels (51-74 g/L) is comparable to the values reported previously for adult racing camels [9] (59-64 g/L) and [10] (53-78 g/L). However, the range obtained in the current study is lower than that reported by Bogin [11] (63-88 g/L). The mean value reported for young camels (54.8 ± 4.0 g/L) is within the reference range reported for young camels at the age of 1 year [12] (49-85 mmol/L). The variations in the concentration of serum protein are generally associated with alterations in the nutritional status of the animals. In lactating female camels, the higher mean value of serum-[Protein] (63.7 g/L) is clearly attributed to the higher concentration of γ-globulins observed (26% = 16.8 g/L) (Table 1).

The current data indicate that albumin represented the main fraction of serum proteins determined by CE in all experimental groups (50%-60%, Table 1). The globulin fractions, α_1 and α_2, β and γ represented about 3%-4%, 9%-10%, 10%-12% and 18%-26%, respectively (Table 1). These findings are higher than that the values reported for young and adult camels [5]. Furthermore, the present results indicate that there was a significant difference between the adult male, female and young camels in the fraction of albumin, α_1, α_2, and γ-globulin. The variation in these values can be considered as age-dependent relationship between the groups.

5. Conclusions

The present study indicates that the serum electrophoresis pattern of the camels is influenced by age. The differences between the current results and values reported previously in the literature could be related to genetic and various environmental factors

including the nutritional status of the animals. The data could be utilised for clinical monitoring of dysprteinaemias in camels.

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Structure–Activity Relationships in the Chelation Activity by Derivatives of 1,2-Dithiole-3-Thiones

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Abstract: The structure and electronic properties of a series of biologically active dithiolethiones (1) have been calculated using semi-empirical. Multi-linear regression analysis suggests that there is a reasonable correlation between the experimental activity of the derivatives against chelation activity and calculated properties such as the HOMO energies, molar refractivity, dipole moments and experimental partition coefficient. From the derived QSAR equations the 3-Methylthio-4p-Tolyle-1,2-Dithiolylium accompanying ion ($CH_3SO_4^-$) and 4-para-tolyl-1,2-dithiole-3-thione (2b and 2) are predicted to show the highest activity against chelation activity, while 3-Methylthio-5p-methoxy phenyl-1,2-Dithiolylium accompanying ion (I^-) (3a) is predicted to be the least active in line with the experimental results.

Key words: Dithiolethiones, partition coefficient $P_{water/n\text{-}octanol}$, chelation activity.

1. Introduction

The synthesis of novel pharmacologically active molecules with reduced toxicity is of prime interest. Recently, QSAR has gained importance in the field of pharmacological sciences [1]. Quantitative structure activity relationships (QSAR) are predictive tools for a preliminary evaluation of the activity of chemical compounds by using computer-aided models. Quantitative structure activity relationship (QSAR) techniques increase the probability of success and reduce time and cost involvement in drug discovery process [2, 3]. A QSAR is essentially a mathematical equation that is determined from a set of molecules with known activities using computational approaches. The exact form of the relationship between structure and activity can be determined using a variety of statistical methods and computed molecular descriptors and this equation is then used to predict the activity of new molecules.

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Early QSARs pioneered by Hansch and Fujita [4] consisted of relatively small number of molecules of a given chemotype being used to derive a simple linear equation to predict the next molecule in the series to be synthesised.

The advantage of this approach was that the terms in the equation were generally simple and easily interpretable, while the kinds of molecules being predicted were generally very similar to those that were already synthesised, giving the user greater confidence in the model predictions.

In contrast, over the past decade an increasing number of QSARs have been reported based on large, diverse datasets, commonly termed global models, which are considered more reliable at predicting diverse structures than QSARs built on small datasets of low diversity [5, 6].

Molecular properties of chemical compounds are usually correlated with biological activity. This correlation is known as structure-activity relationship (SAR) and many of such studies of dithiolethiones have been reported in the literature [7]. It is well

established that many organosulfur compounds have a pronounced biological activity inter alia as antibiotics, analgesics, antidepressants, anti-inflammatory agents, fungicides and bactericides [8, 9]. For example, Dithiolethiones are found in significant amounts in cruciferous vegetables, and the consumption of these vegetables has been inversely associated with a number of cancers. One of these, Oltipraz, has been used in humans as an antischistosomal agent and exhibit strong chemopreventive effects in experimental animals [10].

Thus, these compounds may be ideally suited to test the neuroprotective efficacy of the Nrf2- ARE cascade *in vivo*. Dithiolethiones are cyclic disulfides with a clear structural resemblance to a-lipoic acid (LA), an extensively studied thiol antioxidant. LA has been shown to penetrate the brain and exert neuroprotective efficacy in a number of *in vivo* models [11]. Zhenquan Jia et al. demonstrated that the redox reaction between D3T and DTT (dithiothreitol) generated superoxide. Superoxide was also formed from the redox reaction of D3T with GSH. These findings demonstrate that D3T reacts with thiols, particularly a dithiol, generating superoxide, which may provide a mechanistic explanation for induction of Nrf2-dependent phase 2 enzymes by D3T [12].

In humans, two dithiolethiones are known to possess pharmacological properties other than cancer chemoprevention. For example, oltipraz was originally used as an investigational drug for the treatment of schistosomiasis. Single oral doses of oltipraz have achieved cure rates of 90% in field trials. 5-(4-Methoxy phenyl)-3*H*-1,2-dithiole-3-thione is used currently as a choleretic and to stimulate salivary secretion and it is marketed as an over-the-counter drug in many countries. Thus, general tolerance of dithiolethiones and their acceptance in humans has been examined. Oltipraz and other dithiolethiones are potent inducers of enzymes involved in the maintenance of the reduced glutathione pools as well as enzymes involved in electrophile detoxication, such as NAD(P)H: quinone reductase (QR), epoxide hydrolase, UDP-glucuronosyl transferase and glutathione *S*-transferase (GST). The enhancement of electrophile detoxification through induction of phase II enzymes has been recognized as a characteristic action of many chemopreventive agents. Oltipraz affords protection against acute and chronic hepatotoxicity [13]. Recent studies [14] have shown that D3T increases the activity of phase II enzymes in the bladder in vivo. It is possible, therefore, that this compound could be useful chemo- protective agent at this site. The prediction of the biological activities of drug candidates is the main focus of many computer-aided drug discovery techniques. The pioneering works of generating quantitative structure-activity relationships were introduced by Hansch and coworkers in the form of MLR models. Since then many different QSAR methods were developed and used successfully in drug design and development. However, the MLR-based methods still remain one of the useful computational techniques in drug development.

Little information is available on the chelation activity of iron by dithiolethiones.

This transition metal ion has a great importance in the generation of oxygen free radicals in living organisms. Chelating agents may inactivate metal ions and potentially inhibit the metal-dependent processes [15]. In order to investigate this possibility, eight dithiolethiones derivatives have been tested for their ability to increase this activity. In these studies, we have calculated the structures and properties of all these molecules in an attempt to develop a quantitative structure – activity relationship (QSAR) which could be used to rationalize their behavior against the chelation of iron and would be useful for predictive purposes.

2. Material and Methods

2.1 Method of Calculation

In our semiempirical calculations we use the

(parametric method 3) PM3, and modified neglect of diatomic overlap (MNDO) methods [16].

The MNDO-PM3 method has proved to be highly reliable for calculating the physical properties of molecules [17].

The structures of the eight dithiolethiones were built from standard geometrical parameters by using model building choice at the calculation start to speed up the computational rates [9], a modeling package based on molecular mechanics.

The resulting optimized structures were then used as initial geometries to localize the global minima following a procedure based on semiempirical energy minimization techniques. The geometry optimization was realized on the SCF level, RHF wave functions were used for singlet state molecules; UHF wave functions were used for triplet state salts dithiolethiones.

We have also employed the conjugate gradient algorithm due to Polak-Ribiére as the minimization technique and a RMS gradient of 0.01 $kJ \cdot mol^{-1}$ to define the termination condition in the iterative process. Polak-Ribiére procedure is a conjugate gradient method using one-dimensional searches and it improves other similar methods (such as Fletcher-Reeves algorithm) by also considering the previous conjugate direction. This technique does not require resetting the conjugate direction and it demands slightly more memory but tends to converge more quickly than the Fletcher-Reeves method [18].

MNDO-PM3 method was used to calculate the physical properties of the dithiolethiones like energy of the HOMO (E_{HOMO}), LUMO (E_{LUMO}) frontier orbitals and Mulliken electronegativity (χ).

Other electronic properties were calculated: total energy (ET), heat of formation (HF); dipole moment (μ). These values were obtained from the molecular package hyperchem 8.0.

The values partition coefficient (log *P*) were determined experimentally [19] with minor changes to ionizable compounds. (The use of buffer solutions in place of water should be considered for such compounds [OCDE].) All the calculated proprieties values are gathered in Table l.

2.2 Fe^{2+} Chelation Assay

The Fe^{2+} chelating ability of both dithiolethiones were determined using a modified method of Minotti and Aust with a slight modification by Puntel et al., [20]. Freshly prepared 1 mM $FeSO_4$ (150 μL) was added to a reaction mixture containing 168 μL of 0.1 M Tris-HCl (pH 7.4), 218 μL of saline and the 1 mL of different concentration of dithiolethiones. The reaction mixture was incubated for 5 min, before the addition of 13 μL of 0.25% 1,10-phenanthroline (w/v).

The absorbance was subsequently measured at 510 nm in a spectrophotometer.

The Fe^{+2} chelating ability was subsequently calculated with respect to the reference (which contains all the reagents without the test sample).

3. Results and Discussion

3.1 Ferrous Ions Chelating Capacity

Iron exists in two distinct oxidation states; ferrous ion (Fe^{2+}) and ferric ion (Fe^{3+}). The ferric ion (Fe^{3+}) is the relatively biologically inactive form of iron.

However, it can be reduced to the active Fe^{2+}, depending on the conditions, particularly pH [21], and oxidized back through Fenton type reactions, with production of hydroxyl radicals; or Haber-Weiss reactions with superoxide anions [22]. The production of these radicals can lead to lipid peroxidation, protein modification and DNA damage.

Also, the production of highly ROS such as superoxide anion radicals, hydrogen peroxide and hydroxyl radicals is also catalysed by free iron through Haber-Weiss reaction: $(O_2^{\bullet -} + H_2O_2 \rightarrow O_2 + OH^- + OH^{\bullet})$ [23]. Free iron is known to have low solubility and a chelated iron (i.e., iron-ligand) complex, such as EDTA-Fe, has greater solubility in solution, which can be contributed solely from the ligand. Furthermore, chelated iron, such as EDTA-Fe,

Table 1 The structure and name of dithiolethiones compounds.

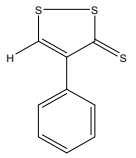
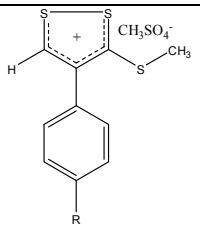
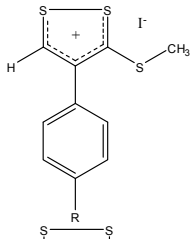
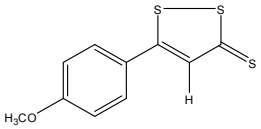
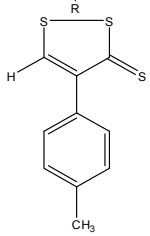
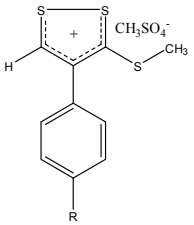
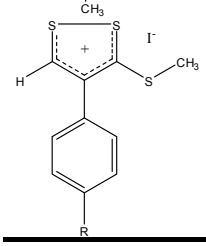
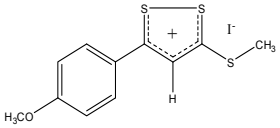
Structure	Name [abbreviation]	Structure	Name [abbreviation]
	4-phenyl-1,2-dithiole-3-thione [1]		3-Methylthio-4p-phenyl-1, 2-Dithiolylium accompanying ion ($CH_3SO_4^-$) (R=H) [1b]
	3-Methylthio-4p-phenyl-1, 2-Dithiolylium accompanying ion (I^-) (R=H) [1a]		5-p-methoxyphenyl-1,2-dithiole -3-thione [3]
	4-p-tolyl-1,2-dithiole-3-thione [2]		3-Methylthio-4p-Tolyle-1, 2-Dithiolylium accompanying ion ($CH_3SO_4^-$) (R=CH_3) [2b]
	3-Methylthio-4p-Tolyle-1, 2-Dithiolylium accompanying ion (I^-) (R=CH_3) [2a]		3-Methylthio-5p-methoxy phenyl-1,2-Dithiolylium accompanying ion (I^-) [3a]

Table 2 Values of selected descriptors used in the regression analysis.

Compound	μ	E_{HOMO}	E_{LUMO}	χ	MR	Log*P*	IC_{50} (mM)
[1]	3.387	-8.938	-2.665	5.801	66.63	3.23	0.510
[2]	3.581	-8.910	-2.640	5.775	70.91	3.49	0.507
[1a]	1.401	-8.626	-2.378	5.502	84.14	1.947	1.240
[2a]	1.706	-8.605	-2.356	5.480	88.43	2.353	2.072
[1b]	14.4	-9.105	-3.972	6.538	81.81	0.45	0.470
[2b]	15.46	-9.030	-4.022	6.526	86.10	1.24	0.749
[3]	5.736	-8.856	-2.601	5.728	74.20	3.82	1.158
[3a]	3.141	-8.242	-2.390	5.316	92.90	2.25	0.758

is also known to be active, since it can participate in iron-catalyzed reactions [22]. Among the transition metals, iron is known as the most important lipid oxidation pro-oxidant due to its high reactivity. The ferrous state of iron accelerates lipid oxidation by breaking down hydrogen and lipid peroxides to reactive free radicals via the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\bullet}$). Fe^{3+} ion also produces radicals from peroxides, although the rate is tenfold less than that of Fe^{2+} ion [24]. Fe^{2+} ion is the most powerful pro-oxidant among various species of metal ions [25].

The chelating of ferrous ions by dithiolethiones was estimated by the phenanthroline assay. Phenanthroline can quantitatively form complexes with Fe^{2+}. In the presence of chelating agents, the complex formation is

inhibited and the red color of the complex fades. Measuring of the color reduction, therefore, it is possible to estimate of the chelating activity of the co-existing chelator [26].

In this assay, the dithiolethiones compound interfered with the formation of the phenanthroline-Fe^{2+} complex, suggesting that it has chelating activity and captures ferrous ions before phenanthroline.

Metal chelating capacity was significant, since it reduced the concentration of the catalysing transition metal in lipid peroxidation.

It was reported that chelating agents are effective as secondary antioxidants because they reduce the redox potential thereby stabilizing the oxidized form of the metal ion.

3.2 QSAR Analysis

3.2.1 Multiple Linear Regressions (MLR)

The statistic technique multiple linear regression is used to study the relation between one dependent variable and several independent variables. It is a mathematic technique that minimizes differences between actual and predicted values. The multiple linear regression model (MLR) was generated using the software Origin Pro, version 8, to predict chelation activities IC_{50} (Eq. (1)).

$$IC_{50} = -42.90068 - 0.07959\mu - 319.9437E_{HOMO} - 315.41091E_{LUMO} - 632.06027\chi + 0.13423MR + 0.30795\log P$$
$$(n = 8,\ R^2 = 0.97,\ F = 6.569) \qquad (1)$$

Four properties were employed to model the activity of the dithiolethiones. These were as follows:

(a) The energies of the frontier molecular orbitals (E_{LUMO} and E_{HOMO}).

The energies of the frontier orbitals are important properties in several chemical and pharmacological processes. The reason for this is the fact that these properties give information on the electron donating and electron accepting character of a compound and, consequently, on the formation of a charge transfer complex (CTC). The energy of the highest occupied molecular orbital (E_{HOMO}) measures the electron donating character of a compound [27] and the energy of the lowest unoccupied molecular orbital (E_{LUMO}) measures its electron accepting character. From these definitions, we have that: (a) the greater the E_{HOMO}, the greater the electron donating capability; (b) the smaller the E_{LUMO}, the smaller the resistance to accept electrons [28].

Both these quantities are directly related to the electron affinity and ionization potential of the molecules in question, though they have positive rather than negative values.

From both these QSAR analyse, it is apparent that the overall chelation effect is associated with a large negative contribution from the E_{HOMO} parameter and a moderating.

For example, taking the value of -9.105 eV for (1b) (Table 2), the relative contributions in Eq. (1) for the E_{HOMO} = + 2914.367.

The chelator doses not only donate electron to the unoccupied d orbital of the metal ion but also accept electron from the d-orbital of the metal leading to the formation of a feedback bond.

A good linear correlation was found between the calculated LUMO energy of the dithiolethiones and the chelation activity.

The electronegativity of the HOMO/LUMO molecular states has been proved as being a reliable fingerprint descriptor for QSAR studies.

The electronegativity plays a critical role in chelation profile of this class of compounds.

(b) Dipole moment (μ).

The calculated dipole moment of the dithiolethiones represents the vector sum of all the atomic charges at each centre. Molecules with large dipole moments are often soluble in polar solvents, such as water, and more likely to chelate through the iron. In contrast, those with small dipole moments have a less polar character, are less soluble in water and less likely to penetrate Fe^{2+}.

The negative coefficient of μ in Eq. (1) indicates that there is a positive correlation between the chelation activity and dipole moment (μ).

This is evidenced by the chelation activity data of substituted dithiolethiones (Table 2) and their μ values. The importance of dipole moment in modulating chelation activity may be due to the presence of methyl sulfate group ($CH_3SO_4^-$) where permanent polarization is seen due to electronegativity difference between the atoms. The sulfur atoms of substituted dithiolethiones may involve in making fruitful binding interactions with Fe^{2+}, through coordination bonding.

(c) Partition coefficient log*P*: Lipophilicity represents the affinity of a molecule or a moiety for a lipophilic environment. It is commonly measured by its distribution behavior in a biphasic system, (e.g., partition coefficient in 1-octanol/water). In the case of dithiolethiones, a statistically significant inverse relationship was observed between chelation activity of substituted dithiolethiones and lipophilic parameter log*P* (Eq. (1)).

(d) Molar refractivity (MR)

The molar refractivity is the molar volume corrected by the refractive index. It represents size and polarizability of a fragment or molecule. The equation Eq. (1) indicate that chelation activity of dithiolethiones derivatives may be decreased with the increase in molar refractivity, Which increase steric/hydrophobic interactions at R4 position negativity contribute for the chelation activity.

Squared correlation coefficient (R^2) of 0.97 in Eq. (1) explains 97% variance in chelation activity. Eq. (1) also indicated statistical significance of > 97 % with *F* value of 6.5697. The R^2 value obtained for MLR method of prediction are 0.99. In order to confirm our results, we have predicted the chelation activity of substituted dithiolethiones using Eq. (1). The comparison of the observed and the predicted values demonstrated that they are close to each other, which is evident by the low residual activity values.

4. Conclusions

The results of the current study demonstrate that the MLR method performs equally well in predicting the chelation activity. Although by just considering the numerical values of R^2 it seems that MLR performs marginally well. In many cases, like the one presented in this work, much simpler and vastly available techniques such as MLR, can predict the property of interest (e.g., chelation activity). The QSAR analysis was available for the dithiolethiones derivatives to predict their chelation activity. The chelation activities of the unknown samples, with dithiolethiones derivatives are easily predicted with QSAR analysis. It is also useful to make a plan to synthesize new compounds, dithiolethiones derivatives, with good biological activities.

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Perception Survey of Carbon Monoxide Risk in Rabat-Salé-Zemmour-Zaër Populations

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Abstract: Carbon monoxide (CO) intoxication is one of the most common types of poisoning in the modern world. To better tailor messages and communication needs to the public, a perception survey of CO and his risk was conducted among the population of Rabat-Salé-Zemmour-Zaer. The authors included 400 people in this survey. The demographic characteristics of the respondents consisted of: 85.0% female, 92.9% adults, 29.7% had a high level of education, 49.6% unemployed, 89.5% were home owners and 53.9% lived in apartments. Water heaters were present in the homes of 91.0% of respondents and 6.5% of them used gas. Gas was used as fuel in 45.4% of cases. For water heaters, 20.8% had an exhaust duct, 9.9% were serviced regularly and 47.5% were installed in a well ventilated area. Regarding the media, television was the medium that allowed 73.4% of the CO to know, regardless of age, sex and level of education among the 94.3% of who ever heard of this deadly gas before the survey.

Key words: Carbon monoxide, poisoning, perception survey.

1. Introduction

Carbon monoxide (CO) continues to be one of the most gas implicated in cases of poisoning involving human damage. In Morocco, although few studies have focused on the role of CO in the toxic condition, some of them have shown that a cause of intoxication far from negligible. CO is an odorless and irritant gas properties, allowing the inhalation of large and potentially lethal without warning symptoms for the victim concentrations. It causes a bastard table and is therefore often under-diagnosed [1, 2].

Carbon monoxide is produced whenever incomplete combustion occurs. CO poisoning can occur in homes, in the workplace, in garages, in a motor vehicle (car, boat, etc.), in a rink, etc.. The primary preventive actions should reduce and secure sourcespotential production of carbon monoxide, as well as ensuring goodventilation conditions. This requires regulatory actions and/or the educational level:

- From product design and materials;
- From the placing on the market;
- Their withdrawal;
- From their installation;
- From maintenance;
- Their use.

Some of these actions (public education, maintenance) may be more effective if they are targeted in the space (risk areas), in time (time at risk, in the year and depending on weather), depending on the population (habitat fragile, elderly, etc.).

Although prevention campaigns poisoning carbon monoxide (CO) are conducted each year by the Poison Control Centre of Morocco (MPCC), this gas is toxic

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causing more acute poisoning in Morocco.

In order to better adapt the messages and communication needs of the public, a perception survey of CO and the risk it represents was conducted among the population of the Rabat-Salé-Zemmour-Zaer. The more health issues and associated institutions were:

- Identify priority areas of communication;
- Provide information to educate players relay preventive actions departmental, regional, national and/or the need for better communication on the subject.

2. Materials and Methods

The study was a descriptive cross-sectional survey. It was conducted among a random sample using the white pages and to extrapolate the results of the study to the entire region of the survey. The tool was used to collect the phone. The survey was conducted between January and February 2012. The questionnaire presented in the appendix focused on socio-demographic and housing characteristics. Environmental and health risk knowledge of CO, as well as information on risk patterns were also collected [3-5].

The Rabat-Salé-Zemmour-Zaer is located in northwest of Morocco, including the capital (Fig. 1).

Fig. 1 Location Rabat-Salé-Zemmour-Zaer throughout the territory.

3. Results

Of the 996 calls, 400 answered the survey (41.6%). Other issues of surveys were divided to refusal to participate, wrong numbers, and unreachable people during the survey period.

The proportion of women estimated from our sample is 84.7%. The age ranges from 15 to 72 years and adult age group is 92.9% (Table 1).

All levels of education are shown in Table 2, respectively, with significant proportion (26.9% and 22.6%) of those with higher education and out of school.

The distribution of the sample estimated by different occupational groups is shown in Table 3, without the professionare the most represented.

Table 1 Structure of the sample by gender and age.

	Percentage
Sex	
Women	84.7
Man	15.0
Age	
[15, 20]	7.1
[20, 74]	92.9

Table 2 Sample structure by level of study.

Level of education	Percentage
Unschooled	22.6
Primary	8.3
Colleges	8.3
High school	24.3
Higher education	26.9

Table 3 Structure of the sample by socio-professional category.

Profession	Percentage
Trading	3.3
Writer	0.3
Eleve	4.3
Student	5.3
Cleaning lady	6.3
Officer	9.0
Engineer	0.3
Reporter	0.3
Doctor	1.0
Nurse	0.3
Employee	0.7
Professor	6.0
Retired	9.3
Unemployed	45.8

A vast majority of people in the region of Rabat-Salé-Zemmour-Zaer own their home and live in a collective dwelling (Table 4).

Individuals report mainly are equipped with water heaters (91.0%), which work with gas (87.0%), the installation locations are shown in Table 5, the majority of these water heaters do not have exhaust duct (79.14%) and are not regularly maintained 90.04%.

The majority of those surveyed do not use heating (68.25%) among those who are equipped with heating, they use an electric heater (23.54%).

As shown in Table 6, the majority of people in the region have been subject of our investigation (70.52%) have heard of CO, and nearly 94.28 people say they know someone who has been the victim of a carbon monoxide poisoning.

Television is the medium that allowed 73.44% know the risk of CO (Table 7).

4. Discussions

During the period of 1991-2011, 18379 cases of CO poisoning were accounted. They represent 4.5% of all poisoning cases in that period of time. These cases increased progressively and were consistent with the overall other poisoning cases (Fig. 2).

Table 4 Structure of the sample according to the type and tenure of housing.

	Percentage
Type of accommodation	
Collective	53.9
Individual	46.1
Occupant status	
Owner	89.5
Tenant	10.4
Number of people occupants	
< 5 people	54.3
> 4 people	45.7
Socio-economic level	
Top	11.2
Middle	43.9
People	44.8
Number of rooms	
< 4 rooms	51.8
> 3 rooms	48.1

Table 5 Structure of the sample by the water heater used.

	Percentage
Water heater	
Yes	91.0
No	9.0
Nature	
Gas	87.0
Electric	11.0
Solar	2.0
Place of installation	
Balcony	13.0
Laundry	10.5
Courtship	5.8
Kitchen	26.4
Garage	0.4
HomeInterior	12.2
Garden	0.4
Bathroom	0.8
Terrace	17.6
Toilet	12.6
Presence of a bleed duct	
Yes	20.8
No	79.2
Maintained	
No	90.1
Yes	9.9

Table 6 Structure of the sample by heating used.

Heating	Percentage
Non	68.2
Yes	31.7
Gas	4.7
Gas + Brazier	0.3
Gas + Fireplace	0.3
Gas + Electric	1.0
Brazier	1.0
Electric	23.5
Solar	0.6

Table 7 Structure of the sample by means of communication.

	Percentage
Have you ever heard of CO	
No	4.3
Yes	70.5
Television	73.4
Neighbor	11.2
Family	8.7
Media	3.7
Newspapers	2.9
Have you ever heard of death by CO	
Yes	94.3
No	5.7

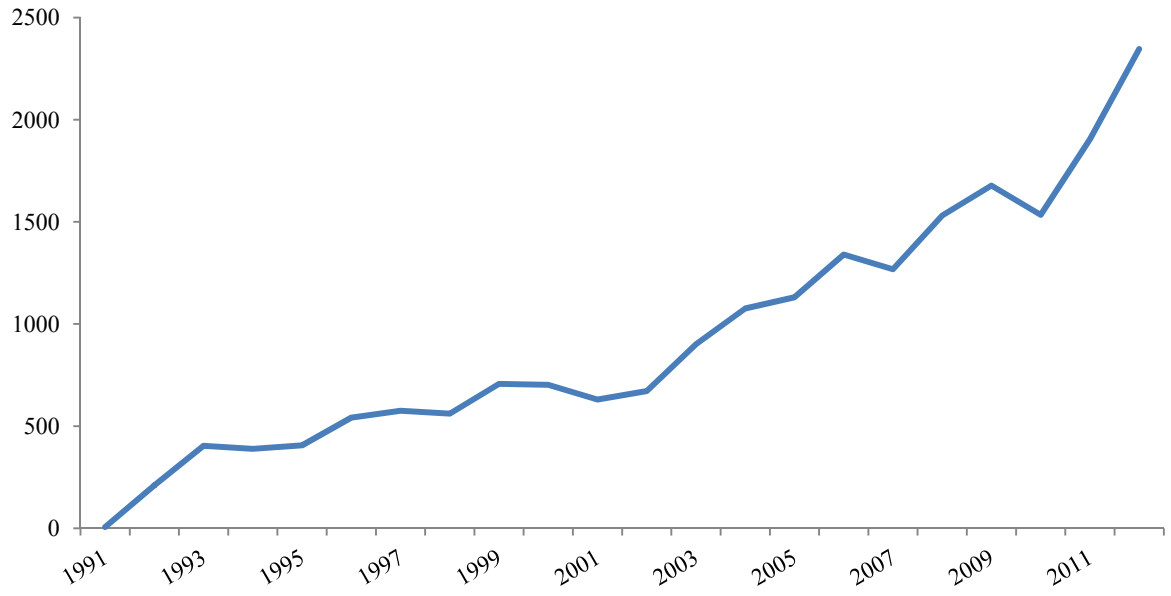

Fig. 2 Trend of CO poisoning, CAPM, 1991-2011.

Using the phone book was the most functional solution to constitute a random sample. The use of white pages led to exclude people who do not have landline. The phone was hung up due to the introductory sentences of the investigation made by the investigator, the investigation was developed to limit the suspicions of commercial approaches highlighted by the Department of Health (see enclosed questionnaire).

The rate of participation in the survey was 49.79%, comparing with a similar survey carried out in lot-et-Garonne in France in 2006 and the participation rate was 41.6 which testimony of interest that leads people to this type of investigation [6]. For each of the demographic variables, the study population is more feminine with an overrepresentation of the bracket adulthood and the majority of respondents have no occupation (45.8%). The highest representation of women and the predominance of no profession is probably due to the fact that the majority of Moroccan women are at home and the fact that our survey was conducted during working weekdays and precisely during working hours. Analysis of data on housing, 53.9% of homes are collective and in 52.4% of cases the water heater is installed in a non-ventilated areas, 79.2% of water heaters do not have a sheath evacuation and 90.1% are not maintained. This could be an explanation for the poisoning occurred oxycarbonees. Although the vast majority of people have had interrogation CO (94.3%) and being aware of a health risk, a much smaller proportion really know the specifications and the signs of intoxication. Knowledge varies by socio-demographic categories. Moreover, without prejudice to the effectiveness of communication used, the results show the need for reflection on the information media [1, 2].

On how to cite most information, television is plebiscite by all, regardless of age, gender and level of study. Other media get scores by socio-demographic variables categories. This will push us to reflect on subsequent to choose specific messages campaigns to target the most vulnerable population and the age and the most appropriate means of communication to raise awareness [7-10].

5. Conclusions

This survey provides information on the risk CO in the general population. It is clear from our study that the knowledge of CO are incomplete. Although the

vast majority of adults in Rabat-Salé-Zemmour-Zaer has heard of CO and is aware of the risk to health, a much smaller proportion actually knows his specifications and signs of intoxication. Thus, despite an equipment rate of households' heater likely CO to produce more than 91%, only a minority knows the gas is undetectable by the senses. The level of knowledge is also related to socio-demographic characteristics, the link the highest being observed with education level (the level of knowledge increases with the level of education). It is therefore important to continue information campaigns this insidious risk that domestic sources and clinical signs partners are more or less known. Regarding potential sources CO, it is essential to further information domestic risks of combustible gas. In addition, information concerning the preferential modes population and without prejudice to the effectiveness of communication these different media, it is interesting to note that some media are favored by the general public so (TV, radio, press). Messages and communication methods should be adapted to the public target prevention activities.

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Questionnaire

Male ☐ Female ☐

Q1: What is your age? .....

Q2: What is your academic level?

Nothing ☐ Preschool ☐ Primary ☐ Preparatory ☐ Secondary ☐ University ☐

Q3: What is the profession? .....

Q4: Are you?

Tenant ☐ Owner ☐

Q5: How many people live in your home (including you)?

.....

Q6: What is the type of your home?

Apartment in a building ☐ Housing individually ☐

A popular neighborhood ☐ Neighborhood average ☐ Neighborhood sophisticated ☐

How many rooms in the house?

.....

Q7: What are the means that you use in your home heating and water heating?

Water heater: Yes ☐ No ☐

What actuation? .....

Where there ..... What kind.....

A ventilation channel: Yes ☐ No ☐

Does maintained by: Yes ☐ No ☐

Canon: Yes ☐ No ☐

Machines that operate with gas heating: Yes ☐ No ☐

Heated (gas, oil and wood): Yes ☐ No ☐

Electric heating: Yes ☐ No ☐

Solar: Yes ☐ No ☐

Other…….

Q8: Do you know someone who was a victim of poisoning heater water or Canon?

……..

……...

Q9: Do you know how limited?

.....

…….

Q10: Have you heard of deaths? .....

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Cartography of Air Pollution in an Industrial City in North-Eastern Algeria by Using Two Indexes: Poleotolerance Index and Atmospheric Purity Index

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Abstract: Of all the methods of studies on bio-estimation of air pollution by lichen flora, the authors cannot justify a choice of one method over another. Consultation of previous work by researchers has allowed us to compare these methods and to better understand their strengths and limitations. Under the terms of estimating the pollution, these methods are classified into three categories. Some are qualitative, quantitative and others are finally some indirect methods. The methods used to assess the overall air pollution relative value for each station studied. As part of our work, the authors have chosen a quantitative approach based on the combination of two methods. They are based on pollution indices obtained from mathematical formulas based on various parameters related to the lichen flora. They are represented by the I.P. (index poleotolerance) and the I.A.P. (index of atmospheric purity) and these two indices allowed us to map of global air pollution in the city of Skikda and petrochemical industry as well as peri-urban areas (Haddaiek, Hamadi Krouma, Hamrouche Hamoudi, Larbi Ben M'Hidi). The authors have identified areas of iso-pollution around different emission centers represented by the largest petrochemical area, traffic and households in urban areas of Skikda, Hamadi Krouma, Hamrouche Hamoudi and Larbi Ben M'Hidi.

Key words: Bio-diversity, bio-indication, lichenic flora, pollution, Algeria.

1. Introduction

The lichen bio-estimation of the air pollution remains substitute and sometimes complementary means to the physical sensors [1, 2]. Of all the methods of studies on the bio-estimation of the air pollution by the lichen flora there is no justification choice of one method over another. The consultation of the previous works realized by Hawksworth [3] and Van Haluwyn [4] allowed to us to compare them and methods to better understand their advantages and their limits. These various approaches contribute to the establishment of a cartography based on iso-pollution areas and classes of lichen species sensitivity on a scale of pollution of a study area. In the case of our works, in the first one the authors used for the first time a quantitative approach called poletolerance index of Strass [5] to assess the degree of air pollution in the city of Skikda and its periphery.

Then, the authors compared the results of this method for the same study area in the I.A.P. (index of atmospheric purity) used by and Fadel & Al Hadjouja [6, 7]. Then, the authors compared the results. A note that these methods allow to assess the air pollution overall relative value for each station studied. They have no direct correspondence with physico-chemical measures of the pollution Steubing [8].

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2. Methods of Study

2.1 Presentation of the Meshing and the Choice of Sampling Stations

The study area is located in a quadriside of 60 km^2. It includes the town of Skikda and peri-urban area, the petrochemical pole, the municipalities of Haddaiek, Hamadi Krouma, Hamrouche Hamadi and Larbi Ben M'Hidi (Fig. 1).

2.2 Establishing Lichen Record

The perimeter of study includes twenty three sampling stations. It is divided into a grid of 40 mesh (1.5 × 1) or 0.003 km and 0.015 gr longitude latitude. The coordinates of each mesh are specified by number in abscissa and by a letter in ordered (Fig. 2). The authors made the statements licheniques in several stations of the zone of study. The authors conducted surveys in several lichen according to the method of Strass [5]. Latter is to consider several trees of different ages and various species. For this four readings on each tree were necessary. Two have been made on the face exposed to the pollution which one at the base and the other at a height of 1 m to 1.50 m above the ground. The other two are on the opposite face. The systematic determinations of lichens were The authors have established a zoning according to exposure to different emanations of various pollution sources and their location in relation to urban areas and industrial zone. In general way, the choice of stations is influenced by the ecological factors (microclimate, abundance of phorophytes and homogeneity of the vegetable formations) but also by the topographic factors [9-11]. During this research work, the authors have considered a coherent ecological system taking into account isolated stations, represented both by roadside trees or isolated trees and secondarily by citrus orchards and the trees of some urban parks.

made using a binocular magnifying glass and in the microscope and for cutting the thallus. Some reagents such as potassium hydroxide in 10% Lugol, iodine and paraphenyl diamine were used for species identification. For species recognition, the authors

Fig. 1 Location of the study area.

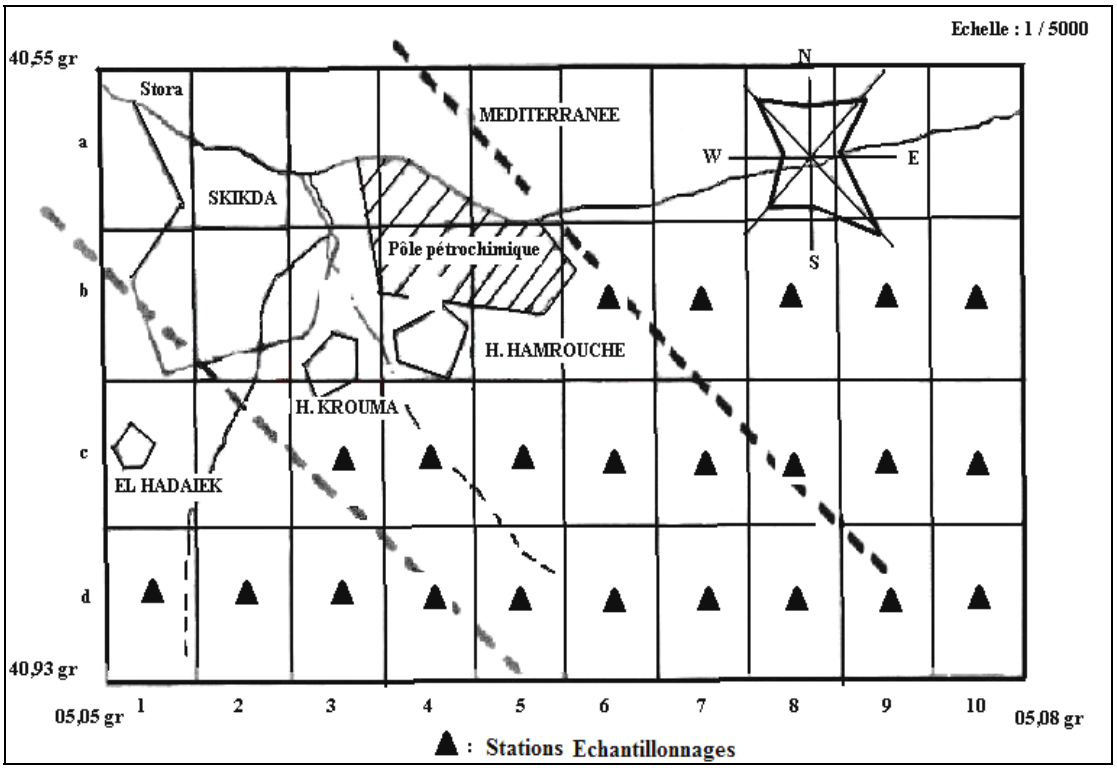

Fig. 2 Sampling stations.

used the lichen flora of Ozenda and Clauzade [12] the guide Jahn [13] and Roux [14]. The authors have identified in our study area 37 species of lichens.

2.3 Calculation of the I.P. (Poleotolerance Index)

The indication I.P. (index of the poleotolerance) is calculated according to the following Eq. (1):

$$I.P. = \sum_{1}^{n} ai \times ci \,/ Ci \qquad (1)$$

n: number of species;

ai: poleotolerance degree of a given species;

ci: degree of coverage of a given species;

Ci: total degree of recovery of all species identified.

3. Results and Discussion

The quantitative value *I.P.* (index poleotolerance) calculated for each station is recorded on Table 1.

By the calculations of the poleotolerance index, the results obtained respectively for every station are appearing globally the air quality each of her. Of these values represented on the Table 1, the authors notice that the stations which have a poleotolerance index the

Table 1 Values of the poleotolerance index of study sites.

Stations	*I.P.* (Poleotolerance index)
b_6	1.4
b_7	0.5
b_8	0.4
b_9	0.34
b_{10}	0.34
c_3	1.8
c_4	5.1
c_5	1.1
c_6	1.1
c_7	1.1
c_8	0.60
c_9	0.60
c_{10}	0.60
d_1	0.50
d_2	0.50
d_3	0.50
d_4	1.25
d_5	0.70
d_6	0.65
d_7	0.60
d_8	0.66
d_9	0.65
d_{10}	0.60

most raised are the most exposed to the atmospheric pollution. It is the case of the stations which have a poleotolerance index included between 0.65 and 5.1. These stations are located in the mesh (b_6, c_3, c_4, c_5, c_6, c_7, d_5, d_6, d_8, d_9). On the other hand, stations situated in mesh (b_7, b_8, b_9, b_{10} c_8, c_9, c_{10}, d_1, d_2, d_3, d_7, d_{10}) which have a poleotolerance index included between 0.34 and 0.60 are the least the exposed to the atmospheric pollution. The card of iso-pollution obtained from the values of the I.P. (poleotolerance index) shows that the stations most highly and moderately polluted are located in the corridor of the prevailing wind of the northwest. The latter is responsible for dispersal and for the distribution of polluants emitted by the various industrial homes in the most polluted stations. Stations that are located near the urban fabric of Skikda and main highways are also more exposed to air pollution. The authors also notice in the same figure as the least polluted stations are practically far from the corridor of the winds northwest and are located far from sources of emanation industrial units and urban areas. These stations are located in mesh Northeast, South-East and South-West.

3.1 Comparison of the Methods

Although the I.P. (poleotolerance index) allows a good translation of the zonation of air pollution around a fireplace emission, which allows precise mapping. It is less sensitive than the A.P.I. (atmospheric purity index) because it is not directly related to the richness of the lichen vegetation [15]. It is for that reason two quantitative methods used in the cartography of the same zone of study. It is I.P. (poléotolérance index) applied in the context of our work to that of I.A.P. (atmospheric purity) used by Metalaoui [16] and Hadjoudja [7]. To enable a better reading result, the authors have shown in Table 2 the values of the I.P. (poleotolerance index) and the I.A.P. (atmospheric purity index).

In general, it appears from the table that the index stations poleotolerance have the lowest of the values of the index of the highest purity air. As it is established well that when the values of the poleotolerance index are low. The atmospheric pollution is useless to weakness. As it is established well that when the values of the poleotolerance index are low, air pollution is absent or low. Contrary to the index of atmospheric purity when its values are elevated air pollution is absent or low. This is true all the more if the authors refer on the scale of atmospheric purity established by Rouidi [17] applied to the same zone of study. The latter determined a scale of the indication index of atmospheric purity. They consider when the index of atmospheric purity is understood between 0 and 5, the stations are polluted; 05 in 10 are averagely polluted; upper to 10 are weakly or not polluted. If the authors compare this scale with the values of the poleotolerance index in Table 2, it really emerges that the least polluted

Table 2 Values of the poleotolerance index of study sites.

	I.P. (Poleotolerance index) $I.P.=\sum_{1}^{n} ai\times ci/Ci$	I.A.P. (Atmospheric purity index) $I.A.P.=1/10.\sum_{1}^{n} Q.f$
b_6	1.4	5.19
b_7	0.5	14.05
b_8	0.4	31.70
b_9	0.34	53.55
b_{10}	0.34	16.90
c_3	1.8	3.20
c_4	5.1	3.90
c_5	1.1	6.24
c_6	1.1	7.52
c_7	1.1	8 .63
c_8	0.60	16.14
c_9	0.60	17.20
c_{10}	0.60	16.45
d_1	0.50	12.58
d_2	0.50	14.85
d_3	0.50	25.78
d_4	1.25	3.50
d_5	0.70	8.25
d_6	0.65	7.10
d_7	0.60	10.06
d_8	0.66	8.51
d_9	0.65	8.46
d_{10}	0.60	10.93

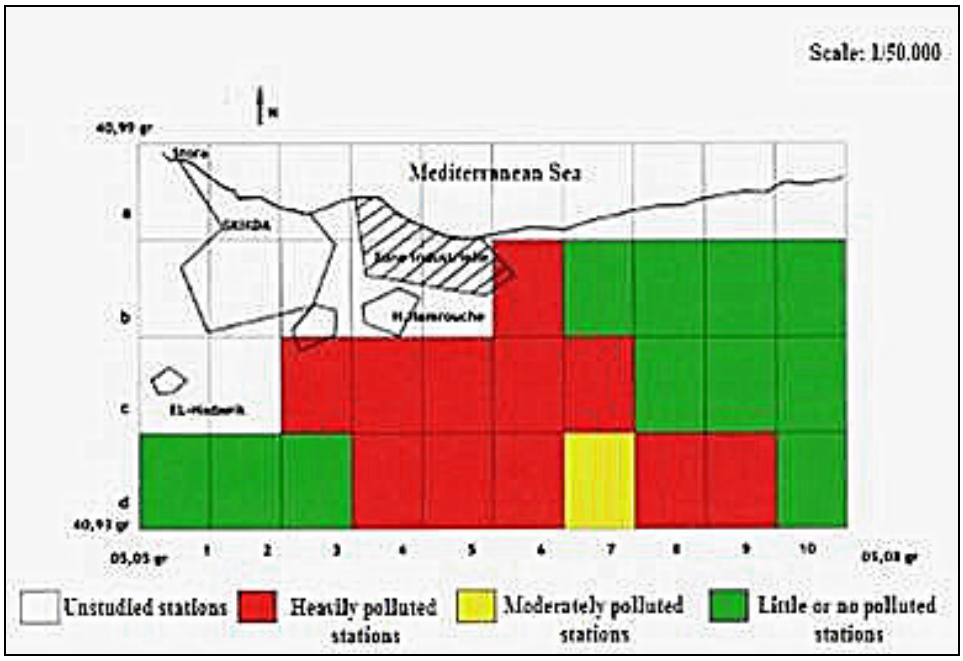

Fig. 3 Map of the air quality of the city of Skikda and its periphery.

stations have low values of the poleotolerance index. This shows well a big similarity of the methods in the cartography of the atmospheric pollution of the studied zone represented in the previous Fig. 3.

4. Conclusions

The results (profits) which the authors obtained allowed us to emit first remark of methodological order. Indeed, the authors note that the use of a biological material of vegetable nature can in any zone devoid of physical analyzers of the atmosphere to measure and to map the region subjected to the pollution by highlighting the zones of iso-pollutions. The results of the index of poléotolérance obtained are respectively for every station created in a global way, the air quality for each of them. The reliability of this method was tested by comparing the zones of iso-pollutions obtained quantitatively with the method of the index of atmospheric purity. The card obtained by the use of the method of the I.P. (index of Poléotolérance) highlighted stations averagely in strongly polluted. They are located in the corridor of pink pollution generated by prevailing winds from the northwest. The latter cross the industrial park and transport pollutants emitted by the various units to deposit them in the stations that are in his direction. The authors also notice that the peripheral stations in the urban fabric are strongly polluted. Stations located near main highways are averagely in strongly polluted. Stations that are weak or non-polluted are located away from sources of emissions of industrial units and urban sources of emissions. They are located outside the corridor, crossed by the prevailing wind of the northwest, and are situated in the meshes of the southeast, the southwest and the northeast. These observations are supported by analytical data of hydrocarbon pollution of the same zone of study [18, 19].

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